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March 18, 2009

Commissioner of Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Re: U.S. Patent Application No.: 10/026,914  
Filed: December 27, 2001  
For: *Allergy Vaccines Containing Hybrid Polypeptides*  
Inventor(s): LINHART, et al.  
Attorney Docket No.: 966927.00006

Sir:

The following documents are forwarded herewith for appropriate action by the U.S. Patent and Trademark Office:

1. Amended Appeal Brief (37 pages; 3-Tab Appendix);
2. Copy of Notification of Non-Compliant Appeal Brief;
3. One (1) return postcard

It is respectfully requested that the attached postcard be stamped with the date of filing of these documents, and that it be returned to our courier.

Respectfully submitted,  
Dobe Law Group, LLC

Christopher E. Aniedobe, Esq.  
Reg. No. 48,293



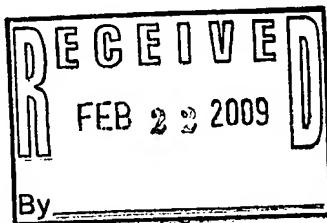
# UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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EXAMINER

ART UNIT PAPER NUMBER

DATE MAILED: 02/18/2009

Please find below and/or attached an Office communication concerning this application or proceeding.



<b>Notification of Non-Compliant Appeal Brief (37 CFR 41.37)</b>	Application No. 10/026,914	Applicant(s) LINHART ET AL.	
	Examiner JaNa Hines	Art Unit 1645	

**--The MAILING DATE of this communication appears on the cover sheet with the correspondence address--**

The Appeal Brief filed on \_\_\_\_\_ is defective for failure to comply with one or more provisions of 37 CFR 41.37.

To avoid dismissal of the appeal, applicant must file an amended brief or other appropriate correction (see MPEP 1205.03) within **ONE MONTH or THIRTY DAYS** from the mailing date of this Notification, whichever is longer.  
**EXTENSIONS OF THIS TIME PERIOD MAY BE GRANTED UNDER 37 CFR 1.136.**

1. ☐ The brief does not contain the items required under 37 CFR 41.37(c), or the items are not under the proper heading or in the proper order.
2. ☐ The brief does not contain a statement of the status of all claims, (e.g., rejected, allowed, withdrawn, objected to, canceled), or does not identify the appealed claims (37 CFR 41.37(c)(1)(iii)).
3. ☐ At least one amendment has been filed subsequent to the final rejection, and the brief does not contain a statement of the status of each such amendment (37 CFR 41.37(c)(1)(iv)).
4. ☒ (a) The brief does not contain a concise explanation of the subject matter defined in each of the independent claims involved in the appeal, referring to the specification by page and line number and to the drawings, if any, by reference characters; and/or (b) the brief fails to: (1) identify, for each independent claim involved in the appeal and for each dependent claim argued separately, every means plus function and step plus function under 35 U.S.C. 112, sixth paragraph, and/or (2) set forth the structure, material, or acts described in the specification as corresponding to each claimed function with reference to the specification by page and line number, and to the drawings, if any, by reference characters (37 CFR 41.37(c)(1)(v)).
5. ☐ The brief does not contain a concise statement of each ground of rejection presented for review (37 CFR 41.37(c)(1)(vi)).
6. ☐ The brief does not present an argument under a separate heading for each ground of rejection on appeal (37 CFR 41.37(c)(1)(vii)).
7. ☐ The brief does not contain a correct copy of the appealed claims as an appendix thereto (37 CFR 41.37(c)(1)(viii)).
8. ☐ The brief does not contain copies of the evidence submitted under 37 CFR 1.130, 1.131, or 1.132 or of any other evidence entered by the examiner **and relied upon by appellant in the appeal**, along with a statement setting forth where in the record that evidence was entered by the examiner, as an appendix thereto (37 CFR 41.37(c)(1)(ix)).
9. ☐ The brief does not contain copies of the decisions rendered by a court or the Board in the proceeding identified in the Related Appeals and Interferences section of the brief as an appendix thereto (37 CFR 41.37(c)(1)(x)).
10. ☒ Other (including any explanation in support of the above items):

The brief does not contain statements and provide reasons as required by 37 CFR 1.192(c)(7) and (c)(8). Appellant must explain why the claims of the group are believed to be separately patentable. Merely pointing out which claims are independent and which claims are dependant is not an argument as to why the claims are separately patentable. Therefore Appellant must explain why the claims of the group are believed to be separately patentable.

/Mark Navarro/  
Primary Examiner, Art Unit 1645



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: LINHART, et al

Serial No.: 10/026,914

Attorney Docket No.: 966927.00006

Filing Date: December 27, 2001

Examiner: Hines, Jana A

Art Unit: 1645

*Allergy Vaccines Containing Hybrid  
Polypeptides*

**AMENDED APPEAL BRIEF**

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to the Notification of Non-Compliant Appeal Brief dated February 18, 2009, requesting Appellants to perfect the Grouping of Claims section and the Summary of Claimed Subject Matter section, cancel "Grouping of Claims" heading and to include a clean copy of the claims involved, Appellants hereby file an Amended Brief in compliance. The above-identified patent application stands before the United States Patent and Trademark Office Board of Appeals and Interferences from the Final Rejection of Claims 42-43, 45-47 by the Examiner in an Official Action mailed June 20, 2007.

Amended Appeal brief being due on or before March 18, 2009, it is not believed that any extension of time is necessary. The Commissioner is hereby authorized to charge any fees which may be required during the entire pendency of the appeal, or credit any overpayment, to Deposit Account 50-4336.

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I. Real Party in Interest:

The real party in interest in the above-captioned application is BIOMAY PRODUKTIONS-und Handels-Aktlengessellschaft Corp. ("Appellant"), a corporation of the Country of Austria, and having a place of business at Lazarettgasse 19, 1090 Vienna, Austria. The application has been assigned of record to SHAN-Beteiligungsgesellschaft mbH by the inventors: Birgit Linhart; Dietrich Kraft; and Rudolf Valenta; said assignee of record having merged with the Real Party in Interest by virtue of a merger agreement executed December 02, 2002.

II. Related Appeals and Interferences:

Appeal is currently pending on US Serial No. 10/026,931. There are no other appeals or interferences known to Appellant or Appellant's legal representative which will directly affect or be directly affected by or have a bearing on the Board's decision in this present appeal.

III. Status of Claims:

Claims 7, 9, 22-25 and 36-47 are pending. Claims 7, 9, 22-25, 36-41, 44 were withdrawn and claims 1-6, 8, 10-21, 26-35, and 48-51 were cancelled. Claims 42-43 were finally rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement, in an Office Action mailed June 20, 2007. Claims 42-43 and 45-47 were finally rejected under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claims 45-47 were finally rejected under 35 U.S.C. §103(a) as being unpatentable over Ball et al., (WO 95/34578) in view of Vrtala et al., (1996. J. Allergy Clin. Immun. Vol. 97(3); 781-787). Claims 42-43 and 45-47 are the subject of the present appeal.



IV. Status of Amendments:

No amendments have been filed subsequent to the Final Rejection. All amendments have been entered and are reflected in the claims appendix.

V. Summary of Claimed Subject Matter:

As recited in independent claim 42, the present invention is directed to a method of preparing fusion polypeptides consisting of timothy grass pollen allergens for use as immunotherapeutic agents; said method comprising the steps of: (a) providing a polynucleotide sequence encoding the fusion polypeptide, See Figure 2. See also Example 2, page 11, paragraphs 2-4, page 12, paragraphs 1-4; (b) introducing said polynucleotide sequence into a host cell, See Example 3, page 13, paragraphs 2-5, page 14, paragraph 1; (c) culturing the host cell obtained in b) under conditions such that the fusion polypeptide is expressed, See Example 3, page 13, paragraph 3; and (d) recovering the expressed fusion polypeptide from the cultured host cell, See Example 3, page 13, paragraph 4; (e) testing the fusion polypeptide as candidate immunotherapeutic agents by administering said polypeptide to a test animal and selecting as immunotherapeutic agents those fusion polypeptides that induce IgE-blocking antibodies and induce stronger immune responses compared with the individual components or fragments thereof, See Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16, paragraph 4, page 17, paragraphs 1-2. Claim 43 depend from claim 42 and further define the use of PCR technology to obtain the polynucleotide sequence encoding the timothy grass pollen polypeptides.

Independent claim 45 recites a pharmaceutical composition comprising one or more fusion allergens of timothy grass pollen allergens for use as immunotherapeutic agents, wherein said agents consists of fusion allergens of timothy grass pollen allergens which have been identified by a method comprising the steps of: (a) providing fusion allergens of naturally occurring timothy grass pollen allergens, See Figure 2. See also Example 2, page 11 paragraphs 2-4, page 12, paragraphs 1-4; (b) challenging an immunological model with said fusion allergens

See Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16, paragraph 4, page 17, paragraphs 1-2; (c) selecting as candidate immunotherapeutic agents, those fusion allergens which induce IgE-blocking antibodies and have reduced allergenic activity compared with the respective allergens which comprise the fusion allergen See Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16 paragraph 4, page 17, paragraphs 1-2.

Independent claim 46 is directed to a hybrid allergen for treatment of IgE-mediated hypersensitivity, wherein said hybrid allergen is a fusion protein consisting of two or more timothy grass pollen allergens. See Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16, paragraph 4, page 17, paragraphs 1-2. Claim 47 depends from claim 46 and is directed to a pharmaceutical composition comprising fusion proteins selected from the group consisting of timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6.

Prior to the claimed invention, allergen-specific immunotherapy was based on systemic administration of crude, ill-defined, non-standardizeable allergens extracts to induce allergen-specific unresponsiveness a great risk for anaphylactic side effects and without regards to the specific sensitization profile of a given patient. The claimed invention, among other things, has overcome the problems of the prior art related to the use of non-standardizeable crude allergen extracts by using allergens of well-defined composition. Furthermore, the inventors have demonstrated for the first time that hybrid fusion polypeptides derived by assembling the epitopes of immunologically distinct allergens can be used for diagnosis and treatment of Type I allergic disorders. See Specification, p.2, paragraph 2. Candidate hybrid polypeptides can comprise at least one complete allergen protein; or two different complete allergen proteins

coming from same or different sources. See Specification, p2, paragraph 6. The claims under consideration are, however, limited to hybrid allergens derived from timothy grass pollen allergens and encompass a method of preparing hybrid fusion timothy grass pollen polypeptides and pharmaceutical compositions directed thereto for use in allergy immunotherapy.

VI. Grounds of Rejection to be Reviewed:

Issue 1

Whether claims 42 - 43 comply with the written description requirement under 35 U.S.C. §112, first paragraph.

Issue 2

Whether claims 42-43 and 45-47 are indefinite under 35 U.S.C. §112, second paragraph as allegedly failing to particularly point out and distinctly claim the subject matter with applicant regards as the invention.

Issue 3

Whether claims 45-47 are unpatentable under 35 U.S.C. §103(a) over Ball et al., (WO 95/34578) in view of Vrtala et al., (1996. J. Allergy Clin. Immun. Vol. 97(3); 781 -787).

VII. Grouping of Claims:

There are three groups of claims, which stand or fall separately. Group 1 consists of independent claim 42 and dependent claim 43. Group 2 consists of independent claim 45. Group 3 consists of independent claim 46 and dependent claim 47.

VIII. Argument:

Issue 1

**Whether claims 42 - 43 comply with the written description requirement under 35 U.S.C. §112, first paragraph.**

The Examiner recognized that claims 42-43 are drawn to a **method** of preparing fusion polypeptides consisting of timothy grass pollen allergens for use as immunotherapeutic agents. See June 06, 2005, Final Office Action, page 2. Incongruously, however, the Examiner asserts that “[n]o information, beyond the characterization of a polypeptide having the ability to encode a fusion polypeptide have been provided, which would indicate that applicants did not have possession of the claimed genus of any polypeptide sequences.” *Id.* 3, paragraph 2. The Examiner further asserts that the specification does not contain the disclosure of the structure of **all** polynucleotide sequences that encode a fusion polypeptide, which is within the scope of the claimed genus. The Examiner further asserts that there is no disclosure of the **exact make-up** of the fusion polypeptide nor does the specification provide for the **structure of the polynucleotide**, nor the structural characterization of the **complete sequence**. *Id.* at 4.

Further incongruously, the Examiner asserts that the **method step** regarding selecting as immunotherapeutic agents those fusion polypeptides that induce IgE-blocking antibodies and induce stronger immune responses compared with the individual components or fragments thereof is a mere functional characterization the polynucleotide and “does not describe the polynucleotide itself.” *Id.* at 5.

Further incongruously, the Examiner cites MPEP 2163 as requiring in a method claim, that the disclosure of a biomolecule by functional characteristics, without any disclosed correlation between function and structure is not sufficient characteristics for written description purposes. *Id.* at 5.

Further incongruously, the Examiner asserts that the specification fails to teach what the

(sic) critical nucleic acids can or cannot be modified and still achieve a functional fusion polypeptide and thus fails to enable the skilled artisan to envision the **detailed chemical structure** of the claimed structure of the claimed polynucleotide. *Id.* at 6.

Further incongruously, the Examiner asserts that method steps comprises in relevant part, “providing “a” polynucleotide encoding the fusion polypeptide,” and that “this reads on a single nucleotide as having the ability to encode the fusion polypeptide,” and that the specification did not disclose a single nucleotide having the ability to encode the fusion polypeptide consisting of timothy grass pollen allergens. *Id.* at 7.

The Examiner further asserts that although claim 42 refers to individual components or fragments thereof, that there is no description of the individual components or fragments thereof. *Id.* at 7.

The Examiner summed up this ground for rejection by stating that “the nucleic acid itself is required.” *Id.* at 7.

Responding to arguments presented in the March 27, 2007, Response and Amendments, in which Applicants, had amended the claims to recite “a polynucleotide sequence” in order to obviate the Examiner’s incongruous remarks that “a polynucleotide” reads on “a single nucleotide,” the Examiner stated that the specification had not explicitly defined a “polynucleotide” and thus “a polynucleotide sequence” had no lower limit on the size of the polynucleotide. *Id.* at 9 and 10.

Responding to arguments presented in the March 27, 2007, Response and Amendments in which Applicants directed the Examiner to Figure 2 and Example 2 page 11, paragraphs 2-4, page 12, paragraphs 1-4, as showing the **construction** of recombinant hybrid allergens, the Examiner, inter alia, asserted that the specification does not contain a structural characterization of the complete sequence. *Id.* at 11. Applicant traverses this rejection because the method of preparing fusion polypeptides of timothy grass pollen allergen is described in the specification in such a way as to reasonably convey to one of ordinary skill in the art at the time the application was



filed that the Applicant had possession of the claimed invention as required by 35 U.S.C. §112, first paragraph.

35 U.S.C. §112, first paragraph sets forth in part:

the specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The MPEP summarizes the Federal Circuit's characterization of the written description requirement by stating that, "[a]n applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention."

MPEP §2163 *citing Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572 (Fed. Cir. 1997). Further, the MPEP states that "[p]ossession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was 'ready for patenting' such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention." MPEP §2163 *citing Pfaff v. Wells Electronics, Inc.*, 525 U.S. 55, 68 (1998); *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, (Fed. Cir. 1997), *cert. denied*, 523 U.S. 1089 (1998); and *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206 (Fed. Cir. 1991).

**a. The Claimed Invention is a Methodology and it is Clearly Erroneous to Examine it as if it were a Product Claim**

The Examiner correctly recognizes that claims 42-43 are drawn to a **method** of preparing fusion polypeptides consisting of timothy grass pollen allergens for use as immunotherapeutic agents, but quite incongruously requires that the inventors must disclose the primary structure, down to exact nucleotide or amino acid sequence of representative, if not, all polynucleotides or polypeptides that can be used to practice the invention. Applicants believe that Application of 35 U.S.C. 112, first paragraph, must start with proper construction of the claimed subject matter and inquiry as to whether or not the claimed subject matter is described to patentably enabling details should be based on what is claimed.

A method claim by definition discloses how to accomplish a claimed objective and the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same.

Independent claim 42, is directed to a method of preparing fusion polypeptides consisting of timothy grass pollen allergens for use as immunotherapeutic agents; said method comprising the steps of: (a) providing a polynucleotide sequence encoding the fusion polypeptide, See Figure 2. See also Example 2, page 11, page 11, paragraphs 2-4, page 12, paragraphs 1-4; (b) introducing said polynucleotide sequence into a host cell, See Example 3, page 13, paragraphs 2-5, page 14, paragraph 1; (c) culturing the host cell obtained in b) under conditions such that the fusion polypeptide is expressed, See Example 3, page 13, paragraph 3; and (d) recovering the expressed fusion polypeptide from the cultured host cell, See Example 3, page 13, paragraph 4; (e) testing the fusion polypeptide as candidate immunotherapeutic agents by administering said polypeptide to a test animal and selecting as immunotherapeutic agents those fusion polypeptides that induce IgE-blocking antibodies and induce stronger immune responses compared with the individual components or fragments thereof, See

Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16, paragraph 4, page 17, paragraphs 1-2.

The Examiner is not contending that the Applicants did not have possession of this five-step process; nor is the Examiner contending that these steps do not accomplish the claimed objective; instead the Examiner is contending that the molecularity of the chemical species practiceable with the invention must be disclosed as well. The Examiner keeps insisting that the Applicants must show that they “had possession of the claimed genus of any polypeptide sequences,” or that “the specification must contain the structure of all polynucleotide sequences that encode a fusion polypeptide,” or that the “exact make-up” of the fusion polypeptide be disclosed; whereas all the Applicants are claiming is a method of preparing fusion polypeptides consisting of timothy grass pollen allergens, said method steps adequately and enablingly described in the specification.

The specification is replete with teachings of how to accomplish the claimed methodology exemplified by the major timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6, the disclosure of which constitutes an adequate representation of the genus of timothy grass pollen allergens. Applicants believe that the specification adequately describes a method of preparing a hybrid polypeptide using hybrid polynucleotide sequences encoding said hybrid polypeptide. Reference is made to Figure 2 wherein the method of producing a hybrid polynucleotide of Phl p 5 and Phl p 1 is reduced to drawing. On page 11, Example 2, page 11, paragraphs 2-4, page 12, paragraphs 1-4, Applicants describe in detail how to construct recombinant hybrid allergens. On page 13, Example 3, page 13, paragraphs 2-5, page 14, paragraph 1, Applicants describe how to obtain a recombinant hybrid polypeptide using the recombinant hybrid polynucleotide sequences of Example 2.

The Patent and Trademark Office provides examiners with a set of guidelines to follow when examining patent applications for compliance with the written description requirement called the “Revised Interim Written Description Guidelines Training Material” (“Guidelines”). See 66 Fed. Reg. 1099, 1099-1111 (January 5, 2001). The Guidelines state that “[t]here is a

strong presumption that an adequate written description of the claimed invention is present in the specification as filed.” 66 Fed. Reg. 1099, 1105. Further, the Guidelines state that the, “[w]ritten description for a claimed genus may be satisfied . . . by disclosure of relevant, identifying characteristics, i.e. structure or other physical and/or chemical properties . . . or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus.” *Id.* at 1106. The Guidelines also provide that, “[t]he absence of definitions or details for well-established terms or procedures should not be the basis of a rejection under 35 U.S.C. [§]112, [paragraph] 1, for lack of adequate written description.” 66 Fed. Reg. 1099, 1105. Following the teaching of the Guidelines, the written description requirement is satisfied by either identifying the features of the invention, such as its physical and/or chemical properties or by using well-known terms of art as the Applicants have done in this case.

**b. The Claimed Methodology is Heuristic and the Examiner Failed to Appreciate the Fundamental Nature of the Claims**

By heuristic, Applicants believe that they had in their possession, as at the time of filing this Application, a method of discovering fusion polypeptides consisting of timothy grass pollen allergens that can be used as immunotherapeutic agents against Type I allergies caused by timothy grass pollens.

By definition in this context, a **heuristic** is a technique designed to solve a problem that ignores whether the solution can be proven to be correct, but which usually produces a good solution or solves a simpler problem that contains or intersects with the solution of the more complex problem. A method of making an invention is in and of itself, useful, inventive, and patentable and the Examiner has failed to recognize this fundamental character of the invention.

Prior to this invention, the conventional method of desensitization of allergy patients is

by the use of systemic administration of crude, nonstandardizeable allergen extracts without regards to their exact chemical composition. On the other hand, great amounts of scientific endeavors have also been put forth in the characterization of known allergens, their epitopic mapping; their primary, secondary, and tertiary structure characterization; as well as experimentally onerous substitution, addition, and deletion studies aimed at potentiating natural allergens or derivatives thereof for use as immunotherapeutic agents. It does clearly appear as if the Examiner, informed by the degree of onerosity of those experimentally intensive routes, imposes a standard of 35 U.S.C. 112, first paragraph, that is inappropriate for the claimed invention under review.

Not only have scientists wrestled with the experimentally onerous characterization and potentiation of known allergens for use as immunotherapeutic agents, there is also a need for cross-sensitization of patients across a broad class of allergens by using the least amount of standardizeable allergen derivatives. The thinking prior to the instant invention is that this can be accomplished by systemic administration of a cocktail of standardized allergen derivative.

The invention under consideration is premised on the principle that rather than making, purifying, and standardizing a cocktail of allergen derivatives, that hybrid polypeptides or fusion allergens consisting of those individual allergens or their derivatives can and surprisingly could be used as immunotherapeutic agents. Moreover, these hybrid polypeptides do not necessarily have to come from one plant or animal species in order to impart cross-sensitization across a broad range of allergens. The inventors of the instant invention were first to discover that hybrid polypeptides or fusion polypeptides can be useful immunotherapeutic agents.

The second leg on which this heuristic methodology stands is that rather than go the experimentally undue and onerous route of molecularly characterizing and epitopically mapping, substituting, adding, deleting, and derivatizing these hybrid allergens or fusion polypeptides, that one could simply follow the far less experimentally involved step (e) of the claimed methodology which is testing the fusion polypeptide as candidate immunotherapeutic agents by administering said polypeptide to a test animal and selecting as immunotherapeutic

agents those fusion polypeptides that induce IgE-blocking antibodies and induce stronger immune responses compared with the individual components or fragments thereof, See Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16, paragraph 4, page 17, paragraphs 1-2.

In other words, the inventors now have in their possession, the equivalent of an immunological litmus test used as a heuristic; a technique designed to solve the problem of immunotherapeutic agents that ignores whether the structure of the resulting agent can be proven in the contemporary sense to have its epitopes favorably positioned as such, but which technique produces a good solution or solves a simpler problem that contains or intersects with the solution of the more complex problem. Granted, step (e) involves some amount of experimentation but it is a mere routine, a mere litmus test; not one that can be remotely characterized as undue given the routes which scientists have heretofore followed and it is this failure to appreciate that this invention is in and of itself, a rapid method of discovering immunotherapeutic agents consisting of hybrid or fusion polypeptides, that informs the Examiner's manifestly erroneous application of 35 U.S.C. 112, first paragraph.

**c. The Claimed Methodology is Inherently Self-Validating and the Methodology does not Stand or Fail on the Exact Molecularity of the Hybrid Polypeptides or Derivatives Thereof**

If the Applicants were claiming a product, then adequate inquiry must be had as to whether they had the claimed product in their possession as at the time of filing of the Application. On the other hand, if the Applicants are claiming a method, then the inquiry as to whether they had possession of the method as at the filing of the Application need not exceed the metes and bounds of the claimed method; especially where as here, those metes and bounds have been clearly and concisely delineated.

Applicants are not claiming to be the first to make hybrid polypeptides nor should the method of making such hybrids, which was routinely done and well known to one of skill in the

art be used to burden the Application. Applicants are also not claiming to be the first to derivatize proteins by substitution, deletion, or insertion of whole fragments, nor should the method of making such derivatives, which was routinely done and well known to one of skill in the art be used to burden the Application. The obligation to concisely state what they have invented, invites the obligation to exclude materials which are quite trite while giving notice to one of skill in the art as to exactly what they had possession of.

In regards, therefore, to the heuristic nature of the claimed invention, it is clear and manifest error to insist that guidance as to molecularity of species amenable to the methodology, other than the restrictions appearing on the face of the invention, be provided, where as here, the invention itself is self-validating. In other words, **however derived**, and whatever the molecularity, be it a two amino-acid sequence or five-hundred amino acid sequence, a hybrid polypeptide of timothy grass pollen allergens can now, by virtue of this invention, be for the first time, **routinely tested** as candidate immunotherapeutic agents by administering said polypeptide to a test animal and selecting as immunotherapeutic agents those fusion polypeptides that induce IgE-blocking antibodies and induce stronger immune responses compared with the individual components or fragments thereof, See Example 5, page 15, paragraph 3, page 16, paragraphs 1-3. See also Example 6, page 16, paragraph 4, page 17, paragraphs 1-2.

By teaching for the first time that hybrid allergens can be useful immunotherapeutic agents and by arming investigators with a litmus test for validating the immunotherapeutic utility of hybrid allergens or hybrid derivative allergens, it is manifest error to require *a priori*, that the molecularity of the chemical species amenable to the claimed methodology be the appropriate subject of the first inquiry where as here the steps in the process inherently validate and thus obviate the need for said first inquiry by means of the equivalence of a mere immunological litmus test.

On the basis of the foregoing, particularly the Examiner's failure to properly characterize the invention and the clearly erroneous misapplication of 35 USC 112, first

paragraph, this ground for rejection should be removed.

**A. Group 2 – Claim 45**

Whether claim 45 complies with the written description requirement under 35 U.S.C. §112, first paragraph, is not at issue and it is moot to present arguments directed thereto.

**B. Group 3 – Claim 46 and 47**

Whether claims 46 and 47 comply with the written description requirement under 35 U.S.C. §112, first paragraph, is not at issue and it is moot to present arguments directed thereto.

**Issue 2**

**Whether claims 42-43 and 45-47 are indefinite under 35 U.S.C. §112, second paragraph as allegedly failing to particularly point out and distinctly claim the subject matter with applicant regards as the invention.**

The Examiner rejected claims 42-43 and 45-47 as indefinite under 35 U.S.C. §112, second paragraph as allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

**A. Group 1, Claims 42, 43**



The Examiner asserts that the limitation in claim 42 of “the individual components or fragments thereof” lacks sufficient antecedent basis and that it is unclear what the individual components are nor is it clear what the fragments thereof is referring to.

Applicants believe that the foregoing, particularly with respect to claim 42 being heuristic as it were, and inherently self validating, that fragments of allergenic proteins can be the proper material for claim 42’s methodology. Applicants have also pointed out that a fusion polypeptide is inherently and necessarily an end-to-end fusion of individual polypeptides and thus “the individual components” necessarily mean the polypeptides derived from naturally occurring allergens which have been fused together. Applicants do not believe that one of ordinary skill in the art will have any ambiguity as to whether a fusion polypeptide inherently contains individual components in an end-to-end fashion.

Further, the Examiner asserts that there is insufficient antecedent basis for “the timothy grass pollen polypeptide” in claim 43. Applicants believe, however, that whereas claim 43 depends from claim 42, and whereas claim 42 recites in pertinent part, “a method of preparing fusion polypeptides consisting of timothy grass pollen allergens ...” the antecedent basis is sufficient to support the claimed limitation in claim 43 and this ground for rejection should be dropped.

#### **B. Group II, Claim 45**

Further, the Examiner asserts that the phrase in claim 45 referring to “the respective allergens which comprise the fusion allergen” is unclear. The Examiner further asserts that “it is unclear how the respective allergens which naturally has only one allergen will comprise a fusion allergen” June 20, 2007, Office Action, page 13. With all due respect, Applicants are at a

complete loss as to whether the Examiner did in fact, advert her mind to the teachings of the invention, particularly to Figure 2 of the invention which shows the construction of a Phl p5 and Phl p1 fusion of respective timothy grass allergens, where respective in this case refers to Phl p 5 or Phl p1 each standing alone. If a fusion protein as is commonly understood in the art is an end-to-end joining of two proteins; Applicants do not share the Examiner's sense of ambiguity with respect to how a fusion allergen is respectively comprised of other allergens.

Applicants had argued that inherent components of elements recited have antecedent basis in the recitation of the components themselves. MPEP § 2173.05(e). The MPEP provides an example: "the limitation 'the outer surface of said sphere' would not require an antecedent recitation that the sphere has an outer surface. Again, in a clearly erroneous application of the law, the Examiner asserts that "the doctrine of inherency refers (sic) the express, implicit, and inherent disclosures of a prior art reference may be relied upon in the rejection of claims under 35 U.S.C. 102 or 103." The "doctrine of inherency" relied upon by the Applicant is within 35 U.S.C. 112, Second paragraph jurisprudence and not under 35 U.S.C. 102 or 103. Again, this basis for rejection should be dropped.

### **B. Group III, Claims 46 and 47**

Although the Examiner had rejected claims 42-43 and 45-47 as indefinite under 35 U.S.C. §112, second paragraph as allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention, the Examiner had not pointed out why and how claims 46 and 47 fail to comply with 35 U.S.C. §112, second paragraph. Indeed, the claims are precisely and distinctly directed to treatment of IgE-mediated hypersensitivity using hybrid allergens of fusion proteins of timothy grass pollen allergens. Applicants believe that these

claims are fully compliant with 35 U.S.C. §112, second paragraph, and the asserted ground for rejection does not apply.

### **Issue 3**

**Whether claims 45-47 are unpatentable under 35 U.S.C. §103(a) over Ball et al., (WO 95/34578) in view of Vrtala et al., (1996. J. Allergy Clin. Immun. Vol. 97(3); 781 -787).**

Claims 45-47 stand rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Ball et al. (WO 95/34578) in view of Vrtala et al. (1996. J. Allergy Clin. Immun., Vol. 97(3): 781 - 787). According to the Examiner, Ball et al. teach that the major grass pollen Phl p1 can be part of a hybrid or fusion polypeptide but does not specifically recite using another plant allergenic protein within the hybrid polypeptide. As explicitly admitted by the Examiner, Ball et al. "do not teach fusion proteins consisting of two or more timothy grass pollen allergens." June 20, 2007, Final Office Action, p 16.

However, to cure the deficiency in Ball, the Examiner asserts that Vrtala et al., teach that DNA coding for three major timothy grass pollen allergens representing group I (Phl p1), group II (Phl p2) and group V(Phl p5) was known. Therefore, concludes the Examiner, "it would have been prima facie obvious at the time of applicants' invention to apply Vrtala et al.'s recombinant Phl p1, Phl p2 and Phl p5 to Ball et al.'s pharmaceutical composition or hybrid allergen in order to enhance antigenicity." *Id.* at 17. Applicants will now traverse.

The Examiner appears to be driven by impermissible hindsight to misconstrue the teachings of the prior art in order to manufacture a clearly erroneous basis for obviousness.

35 U.S.C. §103(a) sets forth in part:

[a] patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains.

Section 103 forbids issuance of a patent when “the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.” *KSR Int’l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1734, 82 USPQ2d 1385, 1391 (2007).

The question of obviousness is resolved on the basis of underlying factual determinations including (1) the scope and content of the prior art, (2) any differences between the claimed subject matter and the prior art, (3) the level of skill in the art, and (4) where in evidence, so-called secondary considerations. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 148 USPQ 459, 467 (1966). See also *KSR*, 127 S.Ct. at 1734, 82 USPQ2d at 1391 (“While the sequence of these questions might be reordered in any particular case, the [*Graham*] factors continue to define the inquiry that controls.”) The Court in *Graham* further noted that evidence of secondary considerations, such as commercial success, long felt but unsolved needs, failure of others, etc., “might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented.” 383 U.S. at 18, 148 USPQ at 467.

In *KSR*, the Supreme Court emphasized “the need for caution in granting a patent based on the combination of elements found in the prior art,” *Id.* at 1739, 82 USPQ2d at 1395, and discussed circumstances in which a patent might be determined to be obvious without an explicit

application of the teaching, suggestion, motivation test. In particular, the Supreme Court emphasized that “the principles laid down in *Graham* reaffirmed the ‘functional approach’ of *Hotchkiss*, 11 How. 248.” KSR, 127 S.Ct. at 1739, 82 USPQ2d at 1395 (citing *Graham v. John Deere Co.*, 383 U.S. 1, 12 (1966) (emphasis added)), and reaffirmed principles based on its precedent that “[t]he combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results.” *Id.* The Court explained:

When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, §103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill.

*Id.* at 1740, 82 USPQ2d at 1396. The operative question in this “functional approach” is thus “whether the improvement is more than the predictable use of prior art elements according to their established functions.” *Id.*

The Court explained, “[o]ften, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue.” *Id.* at 1740-41, 82 USPQ2d at 1396. The Court noted that “[t]o facilitate review, this analysis should be made explicit.” *Id.*, citing *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006) (“[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness”). However, “the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.” *Id.*

**a. Ball et al., the primary reference, does not teach, nor motivate, nor suggest, nor does it provide any articulable reasoning with any rational underpinning to support the combination which the Examiner asserts as obvious.**

The Ball et al. reference is equivalent to U.S. Patent No. 6,008,340. Applicants refer the Board to the following excerpt which captures the entire teaching of Ball *et al.* with respect to fusion proteins of Phl p I epitopes and expressible polypeptides.

A fourth aspect of the invention is a recombinant or synthetic protein or polypeptide displaying the antigenicity of a Phl p I epitope, in particular comprising as an essential part a Phl p I epitope of at least one of the sequences set out in SEQ ID NOS: 5, 7 and 9-28. The protein or polypeptides may be fused to an additional polypeptide, such as beta galactosidase, GST or lambda cII protein or **any other polypeptide that can be expressed as a fusion protein in prokaryotic or eukaryotic cells.**

U.S. 6,008,340 Col. 2, In 64 -67; Col. 3, In 1-6. As admitted by the Examiner, Ball et al. do not teach fusion proteins consisting of two or more timothy grass pollen allergens. But under the new KSR regime, the Examiner need not find a teaching, suggestion or motivation; the Examiner may find an articulable reasoning with any rational underpinning to support the combination.

Applicants believe that the use of Ball et al. which teaches the fusion of galactosidase with Phl p I does not rationally support the combination which the Examiner asserts to be obvious. Indeed the fusion of proteins of interest with coordinate proteins which can enhance the expressibility of the fused protein complex is a well known and often utilized technique to boost prokaryotic gene expression. In this case, the protein of interest is fused with galactosidase and inserted downstream the operon promoter sequences in the galactose operon. Then subsequent administration of isopropyl  $\beta$ -thiogalactoside in liquid culture is used to induce the over-expression of galactosidase and the protein of interest fused with it.

The technique of amplifying expression of desirable proteins by fusing them with

expressible proteins is old and well known. The Board is referred to the following teaching from the Ball *et al.* patent.

The Phl p I epitope encoded by clone 98 was expressed as a beta - galactosidase fusion protein in liquid culture (Huynh *et al.*, 1985) and was affinity purified using an anti-beta-galactosidase affinity column. (Promega, Maddison, USA) as described (Vrtala *et al.*, 1993a).

U.S. 6,008,340 Col. 5, In 59 -65. The Huynh *et al.*, (1985) reference cited in Ball *et al.* at least stands for the teaching that the fusion of proteins with expressible proteins is not new and has been the practice since 1985 and Ball *et al.* taught no more than the fusion of Phl p1 epitopes with expressible proteins merely to amplify the expression of, and to aid in isolation of proteins of therapeutic interest.

The rational inquiry under KSR is not whether Ball *et al.* taught the fusion of timothy grass pollen allergen with an expression booster protein; but whether Ball *et al.* can rationally stand for any teaching that fused timothy grass pollen allergens can and do have immunotherapeutic benefits. Prior to the current invention, no one has taught nor suggested that the fusion of hybrid allergens can produce immunotherapeutic agents more desirable than the respective component allergens. That such is the case was indeed a surprise to the inventors who are leading researchers in this area, and is in fact a surprise to anyone who is abreast with the developments in this art area. The Rule 132 declaration submitted prior affirmed the inventor's surprise that fusion proteins of naturally occurring allergens can be used as immunotherapeutic agents and exhibit increased immunogenicity. See Rule 132 declaration dated September 28, 2004. That Graham factor of surprise coupled with long felt obvious need to cross-sensitize patients against a broad spectrum of allergens with administration of the least amount of immunotherapeutic agents capable of inducing anaphylactic side effects, all tend to negate a rational underpinning for the use of Ball *et al.* against the current invention.

b. Vrtala *et al.*, the secondary reference, does not teach, nor motivate, nor suggest, nor does it provide any articulable reasoning with any rational underpinning to support the combination which the Examiner asserts as obvious.

The Examiner asserts that Vrtala et al. teach that “fusion polypeptides do not significantly affect the allergens IgE-binding capacity (page 782, col.1).” June 20, 2007, Office Action p 16. The Examiner further asserts that Vrtala et al., “teach protein having at least two timothy grass pollen allergens.” Id. at 16. Applicants respectfully traverse.

Either the Examiner did not advert her mind closely to the teachings of Vrtala et al., or she is erroneously applying impermissible hindsight to reconstruct Vrtala et al. in order to tease out an articulable rationale to support the combination which the Examiner asserts as obvious.

Applicants draw the Boards attention to the abstract where Vrtala et al. teaches:

Methods: The three recombinant grass pollen allergens were expressed at high levels in E. Coli as recombinant **nonfusion proteins**, purified by conventional protein chemical methods and tested for their IgE-binding capacity ...

Vrtala et al. p. 781, Abstract. When the Examiner asserted that Vrtala *et al.* teach that “fusion polypeptides do not significantly affect the allergens IgE-binding capacity (page 782,col. 1)”, the Examiner apparently did not advert her mind to the teaching on page 781, col. 2 that “in a plaque lift technique  **$\beta$ -galactosidase fusion proteins** of the above mentioned grass pollen allergens were successfully used to diagnose grass pollen allergy ...” Nor did the Examiner advert her mind to the teaching on page 782, col. 1 that “previous assays were done with recombinant  **$\beta$ -galactosidase fusion allergens**, which in addition to the mature protein contained the leader peptides and a large portion of  $\beta$ -galactosidase.”

Vrtala et al. on page 782, col. 1, were apparently concerned that “although the [B-galactosidase] fused polypeptide did not significantly affect the **allergens’** IgE-binding capacity, the purification protocol had delivered rather small amounts of the recombinant **allergens.**” (Emphasis added) Clearly, Vrtala et al. was speaking in terms of B-galactosidase fused allergens



much like Ball et al.

The rest of Vrtala et al. on page 782 columns 1 and 2 went further to teach how to construct expression plasmids for each of Phl p1, Phl p2, and Phl p 5; expressing them in cell culture using isopropyl B-thiogalactoside as an inducer and the purification of the recombinant allergens. Throughout the disclosure, Vrtala et al. spoke in terms of a plurality of allergens and Applicants cannot find where the Examiner got the support that Vrtala et al. taught the fusion of timothy grass pollen allergens for use as immunotherapeutic agents.

That fused allergens can have immunotherapeutic benefits was a surprise as earlier mentioned. The Rule 132 declaration submitted prior affirmed the inventor's surprise that fusion proteins of naturally occurring allergens can be used as immunotherapeutic agents and exhibit increased immunogenicity. See Rule 132 declaration dated September 28, 2004. That Graham factor of surprise coupled with long felt obvious need to cross-sensitize patients against a broad spectrum of allergens with administration of the least amount of immunotherapeutic agents capable of inducing anaphylactic side effects, all tend to negate a rational underpinning for the use of Ball et al. against the current invention.

Because the Examiner has not articulated a rational basis for the combination which she asserts as obvious, Applicants believe that this basis for rejection should be dropped.

#### **A. Group 1, Claims 42 and 43**

Whether claims 42 and 43 are unpatentable under 35 U.S.C. §103(a) over Ball et al., (WO 95/34578) in view of Vrtala et al., (1996. J. Allergy Clin. Immun. Vol. 97(3); 781 -787) is not at issue and arguments directed thereto are moot.

## CONCLUSION

In view of the forgoing discussion, it is respectfully submitted that the Examiner's rejections of claims 42 to 43, 45 - 47 (Groups 1 to 4) are improper and should be reversed by the Board.

Respectfully submitted,

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IX. Claims Appendix

1 – 6. (Canceled).

7. (Withdrawn) A polynucleotide encoding the hybrid polypeptide of claim 1.

8. (Cancelled).

9. (Withdrawn) A cell transfected or transformed with the polynucleotide of claim 7.

10 - 21. (Cancelled).

22. (Withdrawn) A method for treating an allergic disorder comprising administering the pharmaceutical composition of claim 20 to a patient in need thereof.

23. (Withdrawn) A method for inducing tolerance to a given allergen, comprising administering the pharmaceutical composition of claim 20 to a patient in need thereof.

24. (Withdrawn) A method for providing immunity to a given allergen, comprising administering the pharmaceutical composition of claim 20 to a patient in need thereof.

25. (Withdrawn) A method for detecting antibodies against a given allergenic protein in a sample, comprising conducting *in vitro* antibody tests employing the hybrid polypeptide of any one of claims 1 to 6 or conducting *in vitro* or *in vivo* cellular-based tests employing the hybrid polypeptide of any one of claims 1 to 6.

26 – 35. (Cancelled).

36 (Withdrawn) A method of identifying plant hybrid allergens for treatment of IgE-mediated hypersensitivity to the respective wild-type allergens comprising the steps of:

- (a) providing a fusion allergen of naturally occurring plant allergens;
- (b) challenging an immunological model with said fusion allergen;
- (c) selecting as candidate immunotherapeutic agents, those fusion allergens which induce IgE-blocking antibodies and have reduced allergenic activity compared with the respective wild-type allergens.

37. (Withdrawn) The method of claim 36, wherein the hybrid allergen is a fusion protein of two or more wild-type allergens.

38. (Withdrawn) The method of claim 36, wherein the hybrid allergen is a fusion protein of fragments of two or more wild-type allergens.

39. (Withdrawn) The method of claim 36, wherein the hybrid allergen is a fusion protein of fragments of two or more wild-type allergens, and wherein each fragment contains at least eight consecutive amino acids of the wild-type allergen.

40. (Withdrawn) The method of claim 37, wherein the hybrid allergen is a fusion protein of one or more modifications of at least one of the two or more wild-type allergens.

41. (Withdrawn) The method of claim 36, wherein the hybrid allergen is prepared by chemical synthesis.

42. (Previously presented) A method of preparing fusion polypeptides consisting of timothy grass pollen allergens for use as immunotherapeutic agents comprising:

- (a) providing a polynucleotide sequence encoding the fusion polypeptide;
- (b) introducing said polynucleotide sequence into a host cell;
- (c) culturing the host cell obtained in b) under conditions such that the fusion polypeptide is expressed; and
- (d) recovering the expressed fusion polypeptide from the cultured host cell;
- (e) testing the fusion polypeptide as candidate immunotherapeutic agents by administering said polypeptide to a test animal and selecting as immunotherapeutic agents those

fusion polypeptides that induce IgE-blocking antibodies and induce stronger immune responses compared with the individual components or fragments thereof.

43. (Previously presented) The method of claim 42, wherein the polynucleotide sequence encoding the timothy grass pollen polypeptide is obtained using PCR technology.

44. (Withdrawn) A method of treating IgE-mediated hypersensitivity to plant allergens comprising administering to a patient in need of such treatment, a pharmaceutical composition comprising one or more hybrid plant fusion allergens as immunotherapeutic agents, wherein said agents have been identified by a method comprising the steps of:

- (a) providing fusion allergens of naturally occurring plant allergens;
- (b) challenging an immunological model with said fusion allergen;
- (c) selecting as candidate immunotherapeutic agents, those fusion allergens which induce IgE-blocking antibodies and have reduced allergenic activity compared with the respective wild-type allergens.

45. (Previously presented) A pharmaceutical composition comprising one or more fusion allergens of timothy grass pollen allergens as immunotherapeutic agents, wherein said agents consists of fusion allergens of timothy grass pollen allergens which have been identified by a method comprising the steps of:

- (a) providing fusion allergens of naturally occurring timothy grass pollen allergens;
- (b) challenging an immunological model with said fusion allergens;
- (c) selecting as candidate immunotherapeutic agents, those fusion allergens which induce IgE-blocking antibodies and have reduced allergenic activity compared with the respective allergens which comprise the fusion allergen.

46. (Previously presented) A hybrid allergen for treatment of IgE-mediated hypersensitivity, wherein said hybrid allergen is a fusion protein consisting of two or more timothy grass pollen allergens.

47. (Previously presented) The hybrid allergen of claim 46, wherein said hybrid allergen is a fusion protein of two or more proteins selected from the group consisting of timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, and rPhl p 6.

48 - 51. (Canceled).

X. Evidence Appendix

Tab 1 Ball et al. (WO 95/34578); equivalent to U.S. 6,008,340 and was entered in the record by the Examiner on January 04, 2005.

Tab 2. Vrtala et al. 1996. J. Allergy Clin. Immun., Vol. 97(3): 781 - 787), entered into record as part of Information Disclosure Statement filed June 24, 2002.

Tab 3. Declaration under Rule 132 entered into the record as part of Response and Amendment to Office Action filed August 07, 2006.

XI. Related Proceedings Appendix

None.





US006008340A

**United States Patent** [19]

Ball et al.

[11] **Patent Number:** 6,008,340[45] **Date of Patent:** Dec. 28, 1999

[54] **RECOMBINANT ALLERGEN, FRAGMENTS THEREOF, CORRESPONDING RECOMBINANT DNA MOLECULES, VECTORS AND HOSTS CONTAINING THE DNA MOLECULES**

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**PCT Pub. Date:** Dec. 21, 1995

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[52] **U.S. Cl.** ..... 536/23.6; 536/23.1; 530/300; 530/350; 435/320.1; 435/252.3; 435/254.11; 435/325; 435/410

[58] **Field of Search** ..... 536/23.1, 23.6, 536/24.1; 436/69.1, 69.7, 172.3, 252.3, 254.11, 320.1, 325, 410; 530/300, 350, 370

[56] **References Cited****FOREIGN PATENT DOCUMENTS**

WO9203550 3/1992 Japan .  
WO9410314 5/1994 WIPO .  
WO9421675 9/1994 WIPO .

**OTHER PUBLICATIONS**

Freidhoff et al., J. Allergy Clin. Immunol. 78 (1986) 1190-1201.  
Laffer et al., J. Allergy Clin. Immunol. 94 (1994) 689-698.  
Laffer et al., J. Allergy Clin. Immunol. 94 (1994) 88-94.  
Griffith et al., Fed. Eur. Biochem. Soc. 279 (1991) 210-215.  
Perez et al., Journal of Biol. Chem. vol. 265, No. 27 (1995) 16210-16215.

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[57] **ABSTRACT**

A recombinant DNA molecule comprising a nucleotide sequence (I) which codes for a polypeptide displaying the antigenicity of one, two or more of the Phl p I epitope clones (28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114), with the amino acid sequences defined in SEQ ID NOS: 5, 7, 9 and 12-28, and preferably being derived from grasses or monocotyledonic plants, or a nucleotide sequence (II) which hybridizes with such a nucleotide sequence (I) under conditions of high stringency. Polypeptides displaying the antigenicity of one, two or more of the Phl p I epitope clones (28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114). Recombinant expression vectors containing the recombinant molecule and host cells transformed with the vector. Diagnostic methods based on utilizing the polypeptides in immunoassays for humoral antibodies and cellular reactions.

**7 Claims, No Drawings**

# RECOMBINANT ALLERGEN, FRAGMENTS THEREOF, CORRESPONDING RECOMBINANT DNA MOLECULES, VECTORS AND HOSTS CONTAINING THE DNA MOLECULES

This is a national stage application filed under 35 U.S.C. § 317 of PCT/SE95/00724 filed June 1995.

The present invention relates to the title aspects of the major grass pollen allergen Phl p I and IgE-binding epitopes present in this allergen and corresponding haptens. The invention also relates to fragments, including IgE-binding haptens, from other grass and monocotyledonic plant allergens containing the IgE binding epitopes of Phl p I. The invention is primarily concerned with epitopes that normally are found in one or more group I allergens.

## BACKGROUND TO THE INVENTION

Up to 20% of the population in industrialized countries suffer from Type I allergic symptoms (rhinitis, conjunctivitis, asthma bronchiale) (Myamoto et al., 1992). The crosslinking of IgE which is bound to mast cells and basophils via the high affinity receptor FcεRII is the key event leading to release of biological mediators such as histamine (Segal et al., 1977). The crosslinking event by allergens represents, therefore, a potential target for therapy of Type I allergy. Such therapeutical approaches could either use portions of the IgE-molecule or other ligands, to interfere with the binding of IgE to the high affinity Fcε-receptor, or reagents to block the subsequent signal transduction cascade thus preventing the degranulation of mast cells and basophils (Dreskin et al., 1988). An additional possibility for specific therapy would be to use haptens derived from complete allergens which by binding to IgE monovalently could block the crosslinking of IgE (Valenta et al., 1993a). IgE-haptens could also be used to modulate the immune response or to induce tolerance by immunotherapy with a minimum of anaphylactic side effects. Haptens can be obtained from complete allergens by proteolytic digestion. However, this often results in a mixture of fragments and enzymes that are difficult to characterize. Synthesis of peptides based on the amino acid sequence of the allergens, is an alternative approach. Recently a number of cDNAs coding for important allergens (Scheiner et al., 1992) were isolated which can be used to determine IgE-epitopes by molecular biological techniques.

Grass pollen allergy is spread world wide and according to the prevalence of grass pollen allergy it can be expected that 75% of all allergic patients suffer from grass pollen allergy (Freidhoff et al., 1986).

Among the grass pollen allergic patients more than 90% display IgE-reactivity with group I allergens (Freidhoff et al., 1986; Valenta et al., 1992).

The full amino acid sequences and nucleotide sequences of the major grass pollen allergens have been known for some time (timothy grass Phl p I (Laffer et al., 1993), rye grass (*Lolium perenne*) Lol p I (Perez et al., 1990; Griffith et al., 1991; University of Melbourne WO-A-9203550; Brunet C et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, Feb. 18-22, 1995; Lamontagne P et al., International Sym-

posium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, Feb. 18-22, 1995), and from rye from timothy grass (*Phleum pratense*) Sec c I (Laffer et al., unpublished data).

During the priority year the determination of clones 80, 97 and 98 as carriers for a group I conserved IgE binding epitope has been described (Ball et al., 1994a, b, c; Laffer et al., 1994;).

## Definition

The term IgE-hapten identifies short allergen fragments on which only one IgE-antibody with a given specificity is allowed to bind. A real IgE-binding hapten will give no histamine release because it contains the binding site for exclusively one IgE antibody. The term epitope in the context of the present invention refers to an IgE-epitope if not otherwise specified. An epitope may be located on either an IgE-hapten or a longer polypeptide comprising several IgE-binding sites/epitopes. The term IgE preferentially refers to human IgE.

## OBJECTIVES OF THE INVENTION

The objectives of the invention are to provide simple, better and more reliable in vitro and in vivo tests for grass pollen allergy as well as improved therapeutic methods for this disease.

## The invention

A first aspect of the invention is a recombinant DNA molecule comprising a nucleotide sequence (I) which codes for a polypeptide displaying the antigenicity of at least one of the Phl p I epitope clones 28 (SEQ ID NO: 26), 34 (SEQ ID NO: 15), 41 (SEQ ID NO: 24), 42 (SEQ ID NO: 27), 43 (SEQ ID NO: 14), 50 (SEQ ID NO: 18), 52 (SEQ ID NO: 28), 64 (SEQ ID NO: 20), 80 (SEQ ID NO: 5), 85 (SEQ ID NO: 22), 86 (SEQ ID NO: 23), 95 (SEQ ID NO: 17), 97 (SEQ ID NO: 7), 98 (SEQ ID NO: 9), 103 (SEQ ID NO: 19), 108 (SEQ ID NO: 25), 109 (SEQ ID NO: 21), 113 (SEQ ID NO: 12), and 114 (SEQ ID NO: 16) with the amino acid sequences defined in SEQ ID NOS: 5, 7, 9, 12-28 and preferably being derived from grasses or monocotyledonic plants, or a nucleotide sequence (II) which hybridizes with such a nucleotide sequence (I) under conditions of high stringency. The recombinant DNA molecule comprises also degenerate variants of these nucleotide sequences.

The recombinant DNA molecule may also contain a nucleotide sequence which codes for a polypeptide having antigenic crossreactivity and a high degree of homology, preferably >50% such as >60% or >75%, with Phl p I epitopes from grasses or other monocotyledonic plants, preferably those defined by the amino acid sequences given in SEQ ID NOS: 5, 7 and 9-28.

A second aspect of the invention is a recombinant DNA expression vector or cloning system comprising an expression control sequence operatively linked to any of the recombinant molecules defined above.

A third aspect of the invention is a host cell containing a recombinant molecule or vector according to the first or second aspect, respectively.

A fourth aspect of the invention is a recombinant or synthetic protein or polypeptide displaying the antigenicity of a Phl p I epitope, in particular comprising as an essential part a Phl p I epitope of at least one of the sequences set out

in SEQ ID NOS: 5, 7 and 9-28. The protein or polypeptides may be fused to an additional polypeptide, such as  $\beta$ -galactosidase, GST or lambda cII protein or any other polypeptide that can be expressed as a fusion protein in prokaryotic or eukaryotic cells.

In the inventive poly/oligonucleotides and proteins/polypeptides, at least one of the sequences defined in SEQ ID NOS: 5, 7 and 9-28 constitutes an essential part. For the poly-/oligonucleotides this means that each of them should not be longer than half of the DNA sequence coding for the full length Phl p I allergen (SEQ ID NO: 10) and preferably containing a nucleotide sequence coding for at least one Phl p I epitope, such as being present in the Phl p I fragments specified in SEQ ID NOS: 5, 7 and 9-28. The inventive oligo/polynucleotides chains are often shorter than 25% of the DNA coding for the full length Phl p I allergen.

For the inventive proteins and polypeptides "essential part" means that each of them should not be longer than half of the full length Phl p I allergen and preferably also contain at least one Phl p I epitope, such as one or more of the epitopes defined by the fragments of full length Phl p I allergen specified in SEQ ID NOS: 5, 7, 9 and 12-28. The inventive proteins and polypeptides are often shorter than 25% of the full length Phl p I allergen.

By the expression "a polypeptide displaying the antigenicity of at least one of the clones 28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114" is meant any peptide portion displaying at least one epitope defined by these clones and being recognizable immunologically. It can be envisaged that polypeptides exhibiting Phl p I epitopes may be derivatized to carry analytically detectable groups or water-soluble or water-insoluble solid phases suitable for immunoassays of antibodies directed against them, e.g. IgA, IgD, IgE, IgM or IgG antibodies. In aspects of the invention relating to in vitro diagnostics (see below) the inventive peptides may be a) linked to a water-insoluble phase by physical adsorption or a covalent bond, or b) conjugated covalently to an analytically detectable group (label).

The fifth aspect the invention is an in vitro method for diagnosing allergy to plant proteins by determining humoral antibodies directed towards the plant proteins. The allergies concerned are mostly against grass pollen. The relevant antibodies are mostly of the IgE class but IgG antibodies may also give information about the allergy. In general this method comprises contacting a body fluid sample derived from a patient with an inventive polypeptide. The amounts and conditions are selected so that an immune complex between the polypeptide and antibodies in the sample are formed in an amount that is a function of the amount of antibodies in the sample. The immune complex is then measured in a per se known manner.

More specifically a preferred method of the fifth aspect comprises contacting a body fluid sample containing the Igs concerned, e.g. IgG or IgE, with a polypeptide according to the invention and an anti-IgE antibody so as to form the immune complex containing peptide:IgE:anti-IgE. Normally either the peptide or the anti-IgE is linked to a solid phase that is insoluble or insolubilizable in the assay media so that the complex can be separated from the assay media. The determination step in these variants may be performed

by use of an analytically detectable group (label) that either is covalently linked to the anti-IgE antibody (in case the peptide is linked to the solid phase) or to the peptide (in case the anti-IgE antibody is linked to the solid phase). In case IgG antibodies are to be determined anti-IgG replaces anti-IgE.

A sixth aspect of the invention is a method employing measuring, preferably in vitro, the cellular reaction against a Phl p I epitope. The method comprises using a recombinant or synthetic polypeptide as defined for the fourth aspect to stimulate the cellular reaction. Cellular reactions to be measured are histamine release and T cell proliferation (by  $^3\text{H}$  thymidine uptake).

The samples used in the above-mentioned methods are often derived from blood such as whole blood, serum and plasma, although also other body fluids containing Igs may be used (tears etc).

Commonly accepted solid phase forms useful for immunoassays are walls of microtitre wells, spheres, rods, sheets, strips, pads etc. The solid phase may be porous or non-porous. The material in the solid phase may be a polymer selected among polysaccharides and their derivatives, for instance dextran, pullulan, agarose, cellulose etc, or synthetic polymers, preferably vinyl polymers, such as polyacrylamides, polyacrylates, polystyrene, polyvinyl alcohol etc. The polymers in question are often cross-linked, particularly in case the base polymer as such is water-soluble. Examples of analytically detectable groups are isotopes, enzymes, enzyme substrates, fluorophors, haptens, biotin etc.

A seventh aspect of the invention is a method for the treatment of a mammal, such as a human, which has a pollen allergy by administering a therapeutically effective amount of a recombinant or synthetic polypeptide as defined above. Illustrative examples of the therapeutic aspect of the invention are: a) passive therapy of effector organ (nose, conjunctiva, and lung) to prevent mediator release upon subsequent exposition to the complete allergen, and b) use of the peptides as safe tools for active immunotherapy because a single IgE epitope as such do not release mediators so that high doses can be applied. See further in the Discussion part below. The administration routes will be as commonly applied for current hyposensitization. The doses are likely to be in the  $\mu\text{g}/\text{ml}$ -range per kg body weight, i.e. 10-100  $\mu\text{g}$  per kg body weight.

#### EXPERIMENTAL PROCEDURES

A detailed description referring to the procedure for determining one IgE binding epitope encoded by the clones 80, 97 and 98. During the priority year the same method resulted in that further IgE binding epitopes were deduced (clones 28 (SEQ ID NO: 26), 34 (SEQ ID NO: 15), 41 (SEQ ID NO: 24), 42 (SEQ ID NO: 27), 43 (SEQ ID NO: 14), 50 (SEQ ID NO: 18), 52 (SEQ ID NO: 28), 64 (SEQ ID NO: 20), 80 (SEQ ID NO: 5), 85 (SEQ ID NO: 22), 86 (SEQ ID NO: 23), 95 (SEQ ID NO: 17), 97 (SEQ ID NO: 7), 98 (SEQ ID NO: 9), 103 (SEQ ID NO: 19), 108 (SEQ ID NO: 25), 109 (SEQ ID NO: 21), 113 (SEQ ID NO: 12), and 114 (SEQ ID NO: 16)). See SEQUENCES 2 for their specific sequences and positions within the Phl p I allergen. Construction of an epitope cDNA library from the randomly fragmented Phl p I cDNA.

The cDNA fragment coding for Phl p I (Valenta et al., 1992; Laffer et al., 1993) was excised from plasmid pUC 18 and purified by preparative agarose gel electrophoresis (Sambrook et al., 1989; Ausubel et al., 1990). The cDNA was then randomly digested with DNase I (Sambrook et al., 1989; Ausubel et al., 1990) and fragments shorter than 400 bp was isolated by preparative agarose gel electrophoresis. The cDNA fragments were then end repaired with T4 polymerase (Boehringer Mannheim, Germany), linked with 5' phosphorylated 8-mer ECO R I linkers (Schmidheini, Windisch, Switzerland). After Eco R I digestion, linkers were removed using a nick column (Pharmacia Biotech AB, Uppsala, Sweden) and the inserts were ligated into dephosphorylated lambda gt11 arms (Pharmacia Biotech AB, Uppsala, Sweden). The phage DNA was then in vitro packaged using in vitro packaging extracts (Amersham, Buckinghamshire, U.K.). In the recombinant phage particles, the DNA inserts become fused to the gene for  $\beta$ -galactosidase.

IgE-immunoscreening and analysis of epitope clones.

100,000 phages of the Phl p I library were used to infect *E. coli* Y1090 at a density of 5,000 phages per plate (140 mm diameter). The synthesis of recombinant proteins was induced by overlaying the plates with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) soaked in 10 mM IPTG (Huynh et al., 1985). 114 Phl p I epitope clones were isolated using serum IgE from a Phl p I allergic patient and  $^{125}$ I labeled rabbit anti-human IgE (Pharmacia Diagnostics, Uppsala, Sweden) as described (Breiteneder et al., 1989; Valenta et al., 1991; Vrtala et al., 1993a). The epitope clones were then characterized by hybridization with 3 synthetic oligonucleotides spanning the Phl p I cDNA (Oligo A: 5'GGG GGC TTG TCC ACA TCC TTG TAC CCG C3' bp 191-218, oligo B: 5'GGA GAG GTC GAA GTG GTA GGG G3' bp 372-393, Oligo C: 5'CCG CCA CCA CGT CTC CGT CGC CG3' bp 573-595; SEQ. ID. NO.: 1, SEQ. ID. NO.: 2 and SEQ. ID. NO. 3, respectively). Nineteen clones that had hybridized with only one of the oligonucleotides were tested for IgE-binding with sera from 12 Phl p I allergic patients. Clones 80 (SEQ ID NO: 5), 97 (SEQ ID NO: 7) and 98 (SEQ ID NO: 9), bound IgE from most patients and were further tested for IgE-reactivity with sera from 90 grass pollen allergic patients as described (Valenta et al., 1992). For DNA sequence analysis, phage DNA was prepared, the epitope encoding cDNAs were excised with KpnI/SacI, subcloned into plasmid pUC 18 and both DNA strands were sequenced using lambda gt11 forward and reversed primers (Clontech, Palo Alto, USA) with  $^{35}$ S dCTP (Sanger et al., 1977).

Expression and purification of an immunodominant recombinant Phl p I epitope.

The epitope encoded by clone 98 which bound IgE from 40% of 90 grass pollen allergic patients and contained the shortest cDNA fragment was selected for purification. Recombinant clone 98 phage were used to infect lysogenic *E. coli* 1089 (Huynh et al., 1985). The Phl p I epitope encoded by clone 98 was expressed as a  $\beta$ -galactosidase fusion protein in liquid culture (Huynh et al., 1985) and was affinity purified using an anti- $\beta$ -galactosidase affinity column (Promega, Madison, USA) as described (Vrtala et al., 1993a).  $\beta$ -galactosidase was obtained upon infection of *E. coli* Y1089 with empty lambda gt11 phage and purified in the same way.

IgE-binding of natural timothy grass pollen allergens and recombinant Phl p I epitopes.

Natural timothy grass pollen allergens were extracted from timothy grass pollen (Allergon, Valinge, Sweden) (Vrtala et al., 1993b) separated by SDS-PAGE (Laemmli et al., 1970) and transferred to nitrocellulose (Towbin et al., 1979). The recombinant Phl p I epitope (clone 98) and  $\beta$ -galactosidase were purified and also blotted on nitrocellulose. IgE from grass pollen allergic patients was used to detect the nitrocellulose blotted proteins as described (Jarolim et al., 1989) whereas IgE-binding to non-denatured recombinant epitopes was done using plaquelifts of phage clones as described elsewhere (Spitzauer et al., 1993). IgE-binding synthetic peptides which were prepared by Cambridge Research Biochemicals, U.K. was measured by dot blot assays. One hundred nanograms to two mikrogramms peptide per spot were dotted to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Reactive peptides derived from other allergens and reactive sera were included as positive controls.

In vitro histamine release from basophils of patients.

Four grass pollen allergic patients with strong IgE-reactivity to group I grass pollen allergens were selected according to case history, serological testing, using RAST and immunoblotting with natural and recombinant grass pollen allergens and skin-prick test as described (Valenta et al., 1992). After informed consent was obtained heparinized blood samples were taken and granulocytes were prepared by dextran sedimentation (Valenta et al., 1989). Granulocytes were then incubated with increasing doses of natural timothy grass pollen allergens, anti-IgE mAb E-124-2-8 (positive control), the recombinant  $\beta$ -galactosidase fused Phl p I (clone 98) epitope and  $\beta$ -galactosidase (negative control), respectively. Liberated histamine expressed as percentage of total histamine was measured in the cell free supernatants by radio-immunoassay (Immunotech, Marseille, France) (Valenta et al., 1989).

To ensure that IgE-antibodies specific for clone 98 were present in the serum of the patient when histamine release was performed, the supernatants which were obtained from the granulocyte preparation were probed in parallel with nitrocellulose blotted timothy grass pollen allergens and the Phl p I (clone 98) epitope as described (Valenta et al., 1993b). The supernatants were from four grass pollen allergic individuals and from a non-allergic control individual. A buffer control without addition of a supernatant was also run.

## RESULTS

Isolation and characterization of an immunodominant IE-epitope clone from Phl p I.

100,000 phages from the Phl p I epitope library were screened using serum IgE from a grass pollen allergic individual with IgE-reactivity to group I grass pollen allergens. 114 IgE-binding phage clones were obtained and subsequently tested for hybridization with 3 synthetic oligonucleotides spanning the Phl p I cDNA. 19 clones which hybridized with only one oligonucleotide were further tested with serum IgE from 12 different Phl p I allergic patients. All tested patients displayed IgE-reactivity with clone 98 which therefore contained an immunodominant epitope. Clone 80 reacted with ten out of twelve Phl p I reactive patients and clone 97 with eleven out of twelve patients. When tested

with serum IgE from 90 grass-pollen allergic patients which were selected according to case history, RAST (radioallergosorbent test) and skin prick tests, 40% of the tested sera showed IgE-reactivity with clone 98 whereas 35% reacted with clone 80 and clone 97 (data not shown).

The cDNAs of the three immunodominant epitope clones were sequenced and found to code for almost the same portion of the Phl p I molecule. SEQ ID NOS: 5, 7 and 9-28 shows the alignment of the deduced amino acid sequences of the epitope clones with the amino acid sequences of the major grass pollen allergen from timothy grass Phl p I (Laffer et al., 1993). The amino acid of clone 98 SEQ ID NO: 9 was aligned with the amino acid sequences of the other major grass pollen allergens from rye grass (*Lolium perenne*), Lol p I (Perez et al., 1990; Griffith et al., 1991), and from rye from timothy grass (*Phleum pratense*), Sec c I (Laffer et al., unpublished data). It was found that Clone 98 which reacted with IgE from all patients contained a 15 amino acid-immunodominant IgE-epitope of Phl p I which is highly conserved among the other group I allergens and covers amino acid 101-115 of the mature Phl p I protein. The C-terminal portion of the peptide seemed to be critical for IgE-binding because clone 80 SEQ ID NO: 5 and 97 SEQ ID NO: 7 were less frequently recognized. Peptides of 12 amino acids length were synthesized according to the deduced amino acid sequence of Phl p with 3 amino acids overlaps, spanning the complete allergen. No reactivity of the patients' IgE with these peptides could be detected indicating that the critical length for IgE-binding of the clone 98 epitope is in the range between 13-15 amino acids (data not shown). Two sythetic peptides (D: aa 76-87, E: aa 117-129) which flanked the clone 98 IgE-epitope also did not bind IgE (data not shown).

Comparison of different methods for the determination of B-cell epitopes.

Different methods were used to determine IgE-epitopes (=B-cell epitopes) of Phl p I. Overlapping peptides (Geysen et al., 1987) with a length of 12 amino acids which were synthesized according to the deduced amino acid sequence of Phl p I were tested by dot blotting for reactivity with serum IgE from grass pollen allergic patients. In addition ten peptides covering regions of a predicted high antigenicity (Jameson et al., 1988) of the mature Phl p I allergen:

- peptide A: aa 1-17
- peptide B: aa 20-39
- peptide C: aa 44-69
- peptide D: aa 76-87
- peptide E: aa 117-129
- peptide F: aa 136-147
- peptide G: aa 151-159
- peptide H: aa 177-193
- peptide I: aa 199-209
- peptide J: aa 214-237

were also tested for IgE-reactivity with 35 sera from grass pollen allergic patients by dot blotting. None of the peptides tested reacted with IgE from more than 5% of the grass pollen allergic patients (data not shown).

Since the epitope mapping based on the use of synthetic peptides was unsuccessful, a recombinant mapping strategy was used (Mehra et al., 1986). An epitope expression cDNA library was constructed using randomly fragmented Phl p I

cDNA. 114 IgE-epitope clones could be isolated out of 100,000 phages which were screened with IgE from sera of patients. 19 epitope clones containing cDNAs that hybridized only with one oligonucleotide and, therefore, contained small Phl p I fragments were selected. These clones were further tested with sera from 12 different grass pollen allergic patients to determine immunodominant IgE-epitopes. One prominent IgE-epitope encoded by clone 98 was isolated. This sequence was not predicted to be antigenic by computer analysis (Jareson et al., 1988) nor was it identified by testing overlapping synthetic peptides spanning the complete Phl p I molecule (Geysen et al., 1987).

IgE-binding capacity of recombinant Phl p I epitopes. Recombinant Phl p I epitopes were expressed as  $\beta$ -galactosidase fusion proteins and tested for IgE-binding as native and denatured proteins.  $\beta$ -galactosidase produced by lambda gt11 phage without insert was used as a negative control. Native recombinant IgE-epitopes were obtained by overlaying plates of recombinant *E. coli*/phage with IPTG soaked membranes (Valenta et al., 1992) whereas for the assays under denaturing conditions, IgE-epitopes were purified by affinity to anti- $\beta$ -galactosidase antibodies and were separated by denaturing SDS-PAGE (Laemmli et al., 1970) and electroblotted to nitrocellulose (Towbin et al., 1979). Clone 98 bound IgE from all 12 Phl p I allergic patients and when tested with sera from 90 grass pollen allergic patients which were selected according to clinical criteria (case history, RAST and skin prick test) 40% were found reactive.

Although clone 98 represented the immunodominant epitope some clones seemed to possess a higher IgE binding capacity. The denatured immunoblotted clone 98 epitope also bound IgE from group I allergic patients. Compared with natural timothy grass pollen extracts a different intensity of IgE-binding was observed. This may be explained by the presence of group V allergens which comigrate with group I allergens in natural extracts.  $\beta$ -galactosidase did not bind IgE in any of the two assays.

Identification of the immunodominant recombinant Phl p I IgE-epitope (clone 98) as a IgE-hapten by in vitro histamine release assays.

The recombinant Phl p I IgE-epitope encoded by clone 98 was tested for its capacity to release histamine from basophils of grass pollen allergic patients. Although all of the three patients displayed distinct IgE-reactivity to the nitrocellulose blotted recombinant Phl p I epitope, no histamine release was observed when the patients granulocytes were incubated with the purified epitope. A dose dependent and specific histamine release was measured when granulocytes from patients were incubated with natural grass pollen allergens and anti-IgE mAb (positive control) whereas no release was obtained upon incubation with  $\beta$ -galactosidase (negative control). One grass pollen allergic patient 98 epitope but with high levels of IgE against group V allergens was also included in the histamine release assays. This patient showed no histamine release with the clone 98 epitope whereas a dose dependent histamine release could be obtained with total grass pollen extracts that contained group V allergens.

## DISCUSSION

The present study demonstrates the efficient determination of IgE-epitopes using recombinant techniques. The

cDNA coding for the major grass pollen allergen, Phl p I (Valenta et al., 1992; Laffer et al., 1993), which is the target for IgE-antibodies of 90% of the grass pollen allergic patients, and thus is the target for IgE-antibodies of up to 75% of all allergic patients (Freidhoff et al., 1986; Valenta et al., 1992) was selected as starting material to construct an epitope expression cDNA library. Using IgE of grass pollen allergic patients, an immunodominant IgE-epitope clone containing a Phl p I peptide having a length of 15 amino acids was isolated.

The described 15 amino acids epitope was not predicted by a computer algorithm (Jameson et al., 1988) nor was it detected by overlapping peptide synthesis technology (Geysen et al., 1987).

The knowledge of IgE-epitopes is of particular importance because release of biological mediators such as histamine during the allergic effector reaction requires a divalent cross-link by allergens of IgE bound to mast cells and basophils (Segal et al., 1977). IgE-haptens derived from allergens contain only one IgE-epitope and thus cannot trigger allergic effector mechanisms unless they are polymerized. The immunodominant IgE-epitope which we derived from the major grass pollen allergen Phl p I was therefore purified and tested for its capacity to induce histamine release from the basophils of grass pollen allergic patients. Although in all experiments histamine release could be induced with natural timothy grass pollen allergens in a dose dependent way, no histamine release could be elicited with the recombinant Phl p I epitope which therefore represents an immunodominant IgE-hapten.

IgE-haptens may be useful for two therapeutical approaches of allergic disease: Haptens could be used to block mast cell- and basophil-bound IgE thereby directly inhibiting mediator release. Synthetic epitopes could be synthesized in large amounts and used directly in the effector organs (lung nasal mucosa, conjunctiva). Such an approach would however require the characterization of many different haptens according to the IgE-binding pattern of the patient. Using recombinant DNA techniques for the characterization of allergens and the determination of IgE-epitopes such an approach may be feasible. In addition, it is expected that due to extensive immunological crossreactivities among most allergens it might be possible to define a limited number of IgE-epitopes (Valenta et al., 1993a). As was demonstrated for Phl p I, IgE-epitopes can be determined by immunoscreening of expression libraries derived from cDNAs of allergens. A representative number of patients can then be tested for IgE-reactivity with recombinant epitope clones to obtain immunodominant structures. In a second step most of the additional relevant IgE-epitopes of each allergen have to be characterized. This might be possible because B-cell epitopes, unlike T-cell epitopes, assemble a larger conformation that also has to be available on the surface of the allergen (Berzofsky et al., 1985; Chothia et al., 1991; Laver et al., 1990). thus it can be assumed that the diversity of B-cell epitopes may be much more restricted than that of T-cell epitopes.

In addition to the blocking of the allergic effector reaction, IgE-haptens could also be used to modulate IgE-responses by applying vaccination strategies which aim at the induction of immunological tolerance. Hyposensitization treat-

ment of Type I allergies which is established world wide requires the increasing application of allergens by injection or oral administration. Although hyposensitization is used successfully since 1911 (Noon et al., 1911), many patients undergoing this therapy suffer from severe side effects such as anaphylactic shock. The use of IgE-haptens derived from the allergens might contribute considerably to the improvement of this therapy by reduction of such side effects. Different other methods to modulate the IgE-response in allergic patients which are currently developed might also take advantage from the use of IgE-haptens.

In conclusion our study demonstrates that by use of recombinant techniques an immunodominant IgE-hapten from the major timothy grass pollen allergen Phl p I could be obtained. This allergen was selected as a model allergen because a high proportion (>90%) of all grass pollen allergic patients, and thus almost 75% of all allergic patients show IgE-reactivity with this molecule. Although the obtained 10 amino acids epitope bound IgE from approximately 40% of grass pollen allergic patients, it did not release histamine from the basophils of patients and may therefore be considered as a hapten. We are aware that in addition to the described Phl p I epitope certainly more epitopes from the same molecule and other allergens must be defined to block histamine release by saturating mast cells and basophils of allergic patients. However, the results encourage to continue with the characterization of IgE-haptens for their future use in specific therapy of allergic diseases.

#### REFERENCES

1. Ausubel F M, In: Current protocols in Molecular Biology, Wiley, New York (1990)
2. Ball T et al., J. Biol. Chem. 269 (1994) 28323-28328
3. Ball et al., XV Annual Meeting of the European Academy of Allergology and Clinical Immunology, Stockholm, Sweden, Jun. 26-Jul. 1, 1994
4. Ball et al., 12th European Immunology Meeting, Barcelona, Spain, Jun. 14-17, 1994
5. Brunet C et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, Feb. 18-22, 1995
6. Berzofsky J A, Science 229 (1985) 923-940
7. Breiteneder H et al., EMBO J. 8 (1989) 1935-1938
8. Chothia C, Current Opinion in Structural Biology 1 (1991) 53-59
9. Dreskin S C et al., J. Am. Med. Ass. 260 (1988) 1265-1268
10. Freidhoff L R et al., J. Allergy Clin. Immunol. 78 (1986) 1190-1201
11. Geysen H M et al., J. Immunol. Meth. 102 (1987) 259-274
12. Griffith I J et al., FEBS lett. 279 (1991) 210-215
13. Huynh T V et al., In: cDNA cloning, Oxford, IRL Press, 1 (1985) 49-78
14. Jameson B A et al., CABIOS 4 (1988) 181-186
15. Jarolim E et al., Allergy 44 (1989) 385-395
16. Laemmli U K, Nature (Lond.) 227 (1970) 680-685
17. Laffer S et al., J. Allergy Clin. Immunol. (1993 in press)
18. Laffer et al., 12th European Immunology Meeting, Barcelona, Spain, Jun. 14-17, 1994
19. Lamontagne P et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, Feb. 18-22, 1995
20. Laver W G et al., Cell 61 (1990) 553-556

21. Mehra V et al., Proc. Natl. Acad. Sci. USA 83 (1986) 7013-7017
  22. Miyamoto T, In: Advances in Allergology and Clinical Immunology, Eds Godard P et al., The Parthenon Publishing Group-Carnforth, U.K. and New Jersey, USA, 5 (1992) 343-347
  23. Noon L, Lancet 1 (1911) 1572
  24. Perez M et al., J. Biol. Chem. 265 (1990) 16210-16215
  25. Sambrook J et al., In: Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press (1989) 10
  26. Sanger F et al., Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5468
  27. Ferreira et al., In: Advances in Allergology and Clinical Immunology, Eds. Godard P et al., The Parthenon Publishing Group-Carnforth, U.K. and New Jersey, USA, (1992) 15. 115-126
  28. Segal D M et al., Proc. Natl. Acad. Sci. USA 41 (1977) 457-467
  29. Spitzauer S et al., J. Allergy Clin. Immunol. (1993 in press???) 20
  30. Towbin H et al., E)roc. Natl. Acad. Sci. USA 76 (1979) 4350-4354
  31. Valent P et al., E)roc. Natl. Acad. Sci. USA 86 (1989) 5542-5546
  32. Valenta R et al., Science 253 (1991) 557-560 25
  33. Valenta R et al., Int. Arch. Allergy Appl. Immunol. 97 (1992) 287-294
  34. Valenta R et al., In: Vaccines 93, Eds Ginsberg H S et al., Cold Spring Harbor Laboratory Press, New York, USA (1993a) 37-41 30
  35. Valenta R et al., J. Allergy Clin. Immunol. 91 (1993b) 30
  36. Vrtala S et al., J. Immunol. 151 (1993a) 4773-4781
  37. Vrtala S et al., Int. Arch. Allergy Immunol 102 (1993b) 160-169
  38. University of Melbourne (WO-A-9203550) 35
- SEQUENCES 1. cDNA and deduced amino acid sequence of Phl p I epitope clones discovered up to the priority date c80, (SEQ. ID. NOS.: 4 and 5), c97 (SEQ. ID. NOS.: 6 and 7) and c98 (SEQ. ID. NOS.: 8 and 9).

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c80: CAC ATC ACC GAC GAC AAC GAG GAG CCC ATC GCC CCC TAC CAC TTC  
 H I T D D N E E P I A P Y H F  
 GAC CTC TCC GGC CAC GCG  
 D L S G H A

c97: AAC GAG GAG CCC ATC GCC CCC TAC CAC TTC GAC CTC TCC GGC CAC  
 N E E P I A P Y H F D L S G H  
 GCG TTC GGG  
 A F G

c98: GCC CCC TAC CAC TTC GAC CTC TCC GGC CAC GCG  
 A P Y H F D L S G H A

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55

SEQUENCES 2. Alignment of the deduced amino acid sequences of all Phl p I IgE-epitope clones (SEQ ID NOS: 5, 7, 9 and 12-28) with the full amino acid sequence of the Phl p I allergen (SEQ ID NO: 10). 60

The clone number is at the right end. The full amino acid sequence of the Phl p I allergen is given by the longest lines (SEQ. ID. NO.: 10). The underlined part corresponds to the signal peptide and is not present in the mature polypeptide. 65



MASSSSYLLVVVLFAYFLGSAYGIPKVPPGPNITATYGDKWLDKSTWYGKPTG

IPKVPPGPNITATYGDKWLDKSTWYGKPTG

AGPKDNGGACGYKDVDKPPFSGMTGCGNTPIFKSGRGCSCFEIKCTKPEACSG

AGPKDNGGACGYKDVDKPPFSGMTGCGNTPIFKSGRGC NT

GYKDVDKPPFSGMTGCGNTPIFKSGRGCSCFEIKCTKPEACS 113

KPPFSGMTGCGNT 45

EPVVVHITDDNEEPIAPYHFDLSGHAFGAMAKKGDQKLSAGELELQFRRVKC

PVVVHITDDNE 43

HITDDNEEPIAPYHF 34

HITDDNEEPIAPYHFDLSGHA 80

TDDNEEPIAPYHFDLSG 114

TDDNEEPIAPYHFDLSGHAFGAMA 95

DNEEPIAPYHF 50

NEEPIAPYHFDLSGHAFG 97

EPIAPYHFDLSGH 103

APYHFDLSGHAFGAM 98

KYPEGTRVTPHVEKGSNPNYLALLVKYVNGDGDVVAVDIKERGKDKWIELKESW

HVEKGSNPNVLALLVKYVNGDGDVVAV 64

GAIWRIDTPDKLTGPPTVRYTTEGGTKTEAEDVIPEGWKADTSYESK

LTGPPTVRYTTEGGTKTEAEDVIPEGWKADTSYESK 109

GPPTVRYTTEGGTKTE 85

VRYTTEGGTKTEAEDVIPEGWKADTSYESK 86

RYTTEGGTKTE 41

RYTTEGGTKTEAEDVIPEGWKADTSYESK 108

TTEGGTKTEADV 28

GTKTEAEDVIPEGWKADTSYESK 42

KTEAEDVIPEGWKADTSYESK 52

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#### SEQUENCE LISTING

##### (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 28

##### (2) INFORMATION FOR SEQ ID NO:1:

###### (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

###### (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

###### (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:



-continued

GGGGGCTTGT CCACATCCTT GTACCCGC

28

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGAGAGGTCG AAGTGGTAGG GG

22

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGCCACCAC GTCTCCGTCG CCG

23

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 63 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..63

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAC ATC ACC GAC GAC AAC GAG GAG CCC ATC GCC CCC TAC CAC TTC GAC 48  
 His Ile Thr Asp Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe Asp  
 1 5 10 15

CTC TCC GGC CAC GCG  
 Leu Ser Gly His Ala  
 20

63

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

His Ile Thr Asp Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe Asp  
 1 5 10 15

Leu Ser Gly His Ala  
 20

## (2) INFORMATION FOR SEQ ID NO:6:

-continued

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 54 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..54

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAC GAG GAG CCC ATC GCC CCC TAC CAC TTC GAC CTC TCC GGC CAC GCG 48  
 Asn Glu Glu Pro Ile Ala Pro Tyr His Phe Asp Leu Ser Gly His Ala  
 1 5 10 15  
 TTC GGG 54  
 Phe Gly

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 18 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Glu Glu Pro Ile Ala Pro Tyr His Phe Asp Leu Ser Gly His Ala  
 1 5 10 15  
 Phe Gly

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 33 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 1..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCC CCC TAC CAC TTC GAC CTC TCC GGC CAC GCG 33  
 Ala Pro Tyr His Phe Asp Leu Ser Gly His Ala  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 11 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Pro Tyr His Phe Asp Leu Ser Gly His Ala  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 263 amino acids  
 (B) TYPE: amino acid

-continued

(C) STRANDEDNESS: Not Relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 58..1929

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Met Ala Ser Ser Ser Ser Val Leu Leu Val Val Val Leu Phe Ala Val
 1             5             10             15
Phe Leu Gly Ser Ala Tyr Gly Ile Pro Lys Val Pro Pro Gly Pro Asn
                20             25             30
Ile Thr Ala Thr Tyr Gly Asp Lys Trp Leu Asp Ala Lys Ser Thr Trp
        35             40             45
Tyr Gly Lys Pro Thr Gly Ala Gly Pro Lys Asp Asn Gly Gly Ala Cys
    50             55             60
Gly Tyr Lys Asp Val Asp Lys Pro Pro Phe Ser Gly Met Thr Gly Cys
    65             70             75             80
Gly Asn Thr Pro Ile Phe Lys Ser Gly Arg Gly Cys Gly Ser Cys Phe
        85             90             95
Glu Ile Lys Cys Thr Lys Pro Glu Ala Cys Ser Gly Glu Pro Val Val
    100             105             110
Val His Ile Thr Asp Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe
    115             120             125
Asp Leu Ser Gly His Ala Phe Gly Ala Met Ala Lys Lys Gly Asp Glu
    130             135             140
Gln Lys Leu Arg Ser Ala Gly Glu Leu Glu Leu Gln Phe Arg Arg Val
    145             150             155             160
Lys Cys Lys Tyr Pro Glu Gly Thr Lys Val Thr Phe His Val Glu Lys
        165             170             175
Gly Ser Asn Pro Asn Tyr Leu Ala Leu Leu Val Lys Tyr Val Asn Gly
    180             185             190
Asp Gly Asp Val Val Ala Val Asp Ile Lys Glu Lys Gly Lys Asp Lys
    195             200             205
Trp Ile Glu Leu Lys Glu Ser Trp Gly Ala Ile Trp Arg Ile Asp Thr
    210             215             220
Pro Asp Lys Leu Thr Gly Pro Phe Thr Val Arg Tyr Thr Thr Glu Gly
    225             230             235             240
Gly Thr Lys Thr Glu Ala Glu Asp Val Ile Pro Glu Gly Trp Lys Ala
    245             250             255
Asp Thr Ser Tyr Glu Ser Lys
    260

```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: Not Relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 1..921

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Ile Pro Lys Val Pro Pro Gly Pro Asn Ile Thr Ala Thr Tyr Gly Asp

```

-continued

1	5	10	15
Lys Trp Leu Asp Ala Lys Ser Thr Trp Tyr Gly Lys Pro Thr Gly Ala	20	25	30
Gly Pro Lys Asp Asn Gly Gly Ala Cys Gly Tyr Lys Asp Val Asp Lys	35	40	45
Pro Pro Phe Ser Gly Met Thr Gly Cys Gly Asn Thr Pro Ile Phe Lys	50	55	60
Ser Gly Arg Gly Cys Gly	65	70	

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 43 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Tyr Lys Asp Val Asp Lys Pro Pro Phe Ser Gly Met Thr Gly Cys	1	5	10	15
Gly Asn Thr Pro Ile Phe Lys Ser Gly Arg Gly Cys Gly Ser Cys Phe	20	25	30	
Glu Ile Lys Cys Thr Lys Pro Glu Ala Cys Ser	35	40		

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 13 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Lys Pro Pro Phe Ser Gly Met Thr Gly Cys Gly Asn Thr	1	5	10
---	---	---	----

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 11 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Pro Val Val Val His Ile Thr Asp Asp Asn Glu	1	5	10
---	---	---	----

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 15 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

-continued

His Ile Thr Asp Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe  
 1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 17 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Thr Asp Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe Asp Leu Ser  
 1 5 10 15

Gly

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Thr Asp Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe Asp Leu Ser  
 1 5 10 15

Gly His Ala Phe Gly Ala Met Ala  
 20

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 11 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp Asn Glu Glu Pro Ile Ala Pro Tyr His Phe  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 13 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Glu Pro Ile Ala Pro Tyr His Phe Asp Leu Ser Gly His  
 1 5 10

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 27 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: Not Relevant

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

His Val Glu Lys Gly Ser Asn Pro Asn Val Leu Ala Leu Leu Val Lys  
 1 5 10 15  
 Tyr Val Asn Gly Asp Gly Asp Val Val Ala Val  
 20 25

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Leu Thr Gly Pro Phe Thr Val Arg Tyr Thr Thr Glu Gly Gly Thr Lys  
 1 5 10 15  
 Thr Glu Ala Glu Asp Val Ile Pro Glu Gly Trp Lys Ala Asp Thr Ser  
 20 25 30  
 Tyr Glu Ser Lys  
 35

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Pro Phe Thr Val Arg Tyr Thr Thr Glu Gly Gly Thr Lys Thr Glu  
 1 5 10 15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Val Arg Tyr Thr Thr Glu Gly Gly Thr Lys Thr Glu Ala Glu Asp Val  
 1 5 10 15  
 Ile Pro Glu Gly Trp Lys Ala Asp Thr Ser Tyr Glu Ser Lys  
 20 25 30

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Arg Tyr Thr Thr Glu Gly Gly Thr Lys Thr Glu  
 1                      5                      10

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Arg Tyr Thr Thr Glu Gly Gly Thr Lys Thr Glu Ala Glu Asp Val Ile  
 1                      5                      10                      15

Pro Glu Gly Trp Lys Ala Asp Thr Ser Tyr Glu Ser Lys  
                     20                      25

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Thr Thr Glu Gly Gly Thr Lys Thr Glu Ala Glu Asp Val  
 1                      5                      10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gly Thr Lys Thr Glu Ala Glu Asp Val Ile Pro Glu Gly Trp Lys Ala  
 1                      5                      10                      15

Asp Thr Ser Tyr Glu Ser Lys  
                     20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Lys Thr Glu Ala Glu Asp Val Ile Pro Glu Gly Trp Lys Ala Asp Thr  
 1                      5                      10                      15

Ser Tyr Glu Ser Lys  
                     20

We claim:

1. A recombinant DNA molecule comprising a nucleotide sequence (I) which codes for a polypeptide displaying the antigenicity of one, two or more of the Phl p I epitope clones 45 (SEQ ID NO: 13) 28 (SEQ ID NO: 26), 34 (SEQ ID NO: 15), 41 (SEQ ID NO: 24), 42 (SEQ ID NO: 27), 43 (SEQ ID NO: 14), 50 (SEQ ID NO: 18), 52 (SEQ ID NO: 28), 64 (SEQ ID NO: 20), 80 (SEQ ID NO: 5), 85 (SEQ ID NO: 22), 86 (SEQ ID NO: 23), 95 (SEQ ID NO: 17), 97 (SEQ ID NO: 7), 98 (SEQ ID NO: 9), 103 (SEQ ID NO: 19), 108 (SEQ ID NO: 25), 109 (SEQ ID NO: 21), 113 (SEQ ID NO: 12), and 114 (SEQ ID NO: 16).

2. A recombinant DNA expression vector or expression system comprising an expression control sequence operatively linked to a recombinant molecule defined in any one of claim 1.

3. A host cell containing a recombinant molecule or vector according to claim 1.

4. A polypeptide displaying the antigenicity of at least one of the Phl p I epitopes defined by the amino acid sequences given in clones 45 (SEQ. ID NO: 13) 28 (SEQ ID NO: 26),

34 (SEQ ID NO: 15), 41 (SEQ ID NO: 24), 42 (SEQ ID NO: 27), 43 (SEQ ID NO: 14), 50 (SEQ ID NO: 18), 52 (SEQ ID NO: 28), 64 (SEQ ID NO: 20), 80 (SEQ ID NO: 5), 35 (SEQ ID NO: 22), 86 (SEQ ID NO: 23), 95 (SEQ ID NO: 17), 97 (SEQ ID NO: 7), 98 (SEQ ID NO: 9), 103 (SEQ ID NO: 19), 108 (SEQ ID NO: 25), 109 (SEQ ID NO: 21), 113 (SEQ ID NO: 12), and 114 (SEQ ID NO: 16) or a Phl p I unique portion of these sequences.

5. A polypeptide according to claim 4 in which the polypeptide is fused to an additional polypeptide.

6. A polypeptide according to claim 5 wherein said additional polypeptide is  $\beta$ -galactosidase, GST or lambda cII protein or any other polypeptide that can be expressed as a fusion protein in prokaryotic or eukaryotic cells.

7. The recombinant DNA molecule of claim 1 wherein said nucleotide sequence is derived from grasses or monocotyledonic plants.

\* \* \* \* \*



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# Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens (Phl p 1, Phl p 2, Phl p 5)

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**Background:** Grass pollen allergens belong to the potent elicitors of type I allergy. Approximately 40% of allergic individuals display IgE reactivity with grass pollen allergens. In previous studies we have reported the complementary DNA cloning and expression in *Escherichia coli* of three of the most relevant timothy grass pollen allergens: *Phl p 1*, *Phl p 2*, and *Phl p 5*.

**Objective:** To achieve high level expression of immunologically active timothy grass pollen allergens in *E. coli*, the cDNAs were inserted into expression plasmids.

**Methods:** The three recombinant grass pollen allergens were expressed at high levels in *E. coli* as recombinant nonfusion proteins, purified by conventional protein chemical methods and tested for their IgE-binding capacity by immunoblot and ELISA, as well as in histamine release assays.

**Results:** Milligram amounts of pure recombinant allergens were obtained from cultured *E. coli*. IgE binding to purified recombinant *Phl p 1*, *Phl p 2*, and *Phl p 5* could be demonstrated by immunoblot and ELISA. With ELISAs the percentage of grass pollen-specific IgE directed against the individual recombinant allergens could be estimated. In addition, the purified recombinant timothy grass pollen allergens induced dose-dependent and specific histamine release from patients' blood basophils.

**Conclusion:** Purified recombinant timothy grass pollen allergens represent useful tools for diagnosis and therapy of grass pollen allergy. (*J ALLERGY CLIN IMMUNOL* 1996;97:781-7.)

**Key words:** Recombinant timothy grass pollen allergens, *Phl p 1*, *Phl p 2*, *Phl p 5*, high level expression in *E. coli*, histamine release

Type I allergic reactions caused by grass pollen allergens occur worldwide. Groups of grass pollen allergens with extensive immunologic cross-reactivities have been described in various grass species.<sup>1,3</sup> We have previously isolated complementary DNAs coding for three major timothy grass pollen allergens representing group I (*Phl p 1*),<sup>4</sup> group II (*Phl p 2*),<sup>5</sup> and group V (*Phl p 5*).<sup>6</sup> *Phl p 1* is a target for IgE antibodies in more than 95% of patients with grass pollen allergy. *Phl p 5*, though bound by fewer

## Abbreviations used

BSA: Bovine serum albumin  
DFP: Diisopropyl fluorophosphate  
PBS: Phosphate-buffered saline

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patients (80% of patients with grass pollen allergy) is of particular importance because of its extremely high IgE-binding capacity; and *Phl p 2* represents a low molecular weight allergen (10 kd) for 60% of the patients, and although similar in sequence to *Phl p 1*, shows no relevant immunologic similarity to *Phl p 1*. In a plaque lift technique  $\beta$ -galactosidase fusion proteins of the above-mentioned grass pollen allergens were successfully used to diagnose grass pollen allergy in a representative number of individuals,<sup>7</sup> and it was further demonstrated that recombinant *Phl p 1*, *Phl p 2*, and *Phl p 5*, as well as timothy grass profilin (*Phl p 11*),<sup>8</sup> can be used to precisely determine the sensitization patterns of these patients with

allergy (allergogram).<sup>9</sup> In addition, it was demonstrated that recombinant timothy grass pollen allergens bound a high proportion of grass pollen-specific IgE.<sup>9</sup> However, the previous assays were done with recombinant  $\beta$ -galactosidase fusion allergens, which in addition to the mature protein contained the leader peptides and a large portion of  $\beta$ -galactosidase. Although the fused polypeptide did not significantly affect the allergens' IgE-binding capacity, the purification protocols had delivered rather small amounts of the recombinant allergens. In this study we have modified the allergen-encoding cDNAs so that milligram amounts of functional nonfusion allergens were expressed in *Escherichia coli*.<sup>10</sup> The recombinant grass pollen allergens were tested for antibody binding and specific histamine release and were found to behave similarly to their natural counterparts. A considerable improvement of in vitro and in vivo diagnosis of grass pollen allergy is expected if recombinant allergens can be used for a routine determination of the sensitization patterns in patients with grass pollen allergy. Allergen-specific diagnosis may consecutively improve specific immunotherapy.

## METHODS

### Characterization of patients with timothy grass pollen allergy

Patients allergic to grass pollen in this study were evaluated by case history, RAST (Pharmacia, Uppsala, Sweden) and skin prick test. Sera from allergic individuals had been tested with natural and recombinant timothy grass pollen allergens and were selected from 100 tested sera to reflect a representative binding pattern.<sup>7,9</sup>

### Construction of the expression plasmids for Phl p 1, Phl p 2, and Phl p 5

The cDNA clones of Phl p 1, Phl p 2, and Phl p 5<sup>4-6</sup> were transcribed by polymerase chain reaction to DNA fragments coding for the mature allergens. The signal peptides were replaced by a methionine by placing an *Nde* I (Phl p 1, Phl p 2) or an *Nco* I (Phl p 5) restriction site, both of which contain the ATG codon, in front of the coding region of the mature protein. An *Eco* R I site was introduced immediately after the stop codon. The genes were inserted as *Nde* I/*Eco* R I (Phl p 1, Phl p 2) or *Nco* I/*Eco* R I (Phl p 5) fragments into pMW 172<sup>11</sup> (Phl p 1, Phl p 2) a derivative of pRK 172<sup>12</sup> or pMW 175 (Phl p 5), a derivative of pMW 172 where the *Nde* I site is replaced by *Nco* I. The plasmids were transfected into *E. coli* BL 21 (DE3) with the calcium chloride method.<sup>13</sup> BL 21 is derived from *E. coli* strain B.<sup>14</sup>

### Expression of recombinant allergens in *E. coli*

Recombinant Phl p 1, rPhl p 2, and rPhl p 5 were expressed in *E. coli* BL21 (DE3) in liquid culture on

induction with isopropyl  $\beta$ -thiogalactoside (0.2 to 0.4 mmol/L) when cells were grown to an optical density of 0.8 to 1.2. Yields of 28 mg (Phl p 1), 3 mg (Phl p 2), and 14 mg (Phl p 5) per gram wet weight of cells were obtained in 150 ml cultures. The amount of proteins was estimated from band intensities of Coomassie blue-stained sodium dodecylsulfate polyacrylamide electrophoresis gels (Coomassie blue; Sigma Chemical Co., St. Louis, Mo.).

### Purification of recombinant allergens

**Phl p 1.** Phl p 1 was expressed in inclusion bodies, which were isolated after cells were thawed and suspended in 10 mmol/L Tris (pH 8), 2 mmol/L  $\beta$ -mercaptoethanol, 0.1% vol/vol Triton X-100, 0.5 mmol/L ethylenediaminetetraacetic acid (5 ml buffer/gm cells). Cell lysis was performed by adding lysozyme (20  $\mu$ g/gm cells) for 30 minutes at room temperature.  $\text{Ca}^{2+}$  was then added to 3 mmol/L final concentration, and the mixture was then digested with deoxyribonuclease I (0.1 mg/gm) for an additional 30 minutes at room temperature. The volume was then doubled by addition of 20 mmol/L Tris pH 8, 1% wt/vol desoxycholate, 2 mmol/L ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid, and 0.2 mol/L NaCl (buffer P). The lysate was then centrifuged (5 minutes, 8000 g) and the pellet washed four times with fourfold-diluted buffer P containing 0.05 mmol/L diisopropyl fluorophosphate (DFP) by centrifugation. The resulting pellet was finally washed with 10 mmol/L Tris pH 8, 3% vol/vol isopropanol to remove residual detergents.

Inclusion bodies were then solubilized with 8 mol/L urea, 10 mmol/L Tris pH 8, 1 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L  $\beta$ -mercaptoethanol, 0.05 mmol/L DFP (5 ml/gm cells) and dialyzed against the same buffer without urea. The dialyzed sample was then centrifuged for 10 minutes at 8000 g to remove precipitated material (containing approximately 20% to 30% of the recombinant Phl p 1), and the supernatant was then adjusted to 150 mmol/L NaCl and dialyzed against 100 mmol/L NaCl, 20 mmol/L  $\text{NaH}_2\text{PO}_4$ , 0.05 mmol/L DFP without adjustment of pH. After dialysis, the supernatant was extracted with 0.5% vol/vol of chloroform, and the aqueous phase was precipitated with ammonium sulfate 60% wt/vol. A final purification step with a diethylaminoethyl cellulose column (M. Susani, et al. Unpublished data) yielded a soluble protein that was more than 95% pure.

**Phl p 2.** Phl p 2 was expressed as soluble protein. Cells were lysed as described above and centrifuged for 15 minutes at 14,000 g; the supernatant was brought to 0.05 mmol/L DFP. The supernatant was then dialyzed against 50 mmol/L phosphate buffer, pH 4.5, overnight. After centrifugation at 14,000 g for 15 minutes, the supernatant was brought to pH 5.5 with NaOH, and ammonium sulfate was added to 65% wt/vol. After another centrifugation (14,000 g, 15 minutes), the supernatant was applied to a phenyl-Sepharose column (Pharmacia), and the protein was eluted with 1 mol/L ammonium sulfate. The protein was then dialyzed against 25 mmol/L imidazole, pH 7.4, and rechromatographed on a diethylami-

Source: *Journal of Allergy and Clinical Immunology*

noethyl cellulose-Sepharose column to yield a protein more than 98% pure.

**Phl p 5.** *E. coli* cells were lysed as described above. After centrifugation (15 minutes, 14,000 g), the supernatant was adjusted to pH 6.0 with HCl. Material that was precipitated by addition of ammonium sulfate between 30% and 50% wt/vol was used for further purification. The precipitate was dissolved in 10 mmol/L phosphate, 0.1 mmol/L dithiothreitol, and 0.1% Triton X-100, pH 5.0, then dialyzed against the same buffer without Triton X-100 and applied to SP-Sepharose. Fractions containing more than 90% pure Phl p 5 were concentrated by ammonium sulfate precipitation, dissolved in 100 mmol/L phosphate, pH 5.3, brought to 0.7 mol/L ammonium sulfate and chromatographed on a PS-Sepharose column to yield a protein of more than 99% purity.

#### **IgE binding to natural timothy grass pollen extracts and purified recombinant timothy grass pollen allergens (Phl p 1, Phl p 2, and Phl p 5)**

IgE binding to timothy grass pollen allergens was measured by immunoblotting and ELISA. Nitrocellulose strips, containing total timothy grass pollen proteins, purified rPhl p 1, purified rPhl p 2, or purified rPhl p 5 were incubated with sera from 13 different representative patients with timothy grass pollen allergy. Patients' sera were diluted 1:10 in buffer G (50 mmol/L sodium phosphate, pH 7.5, 0.5% Tween-20, 0.5% bovine serum albumin [BSA], 0.05% NaN<sub>3</sub>), and bound IgE was detected with iodine 125-labeled anti-human IgE (Pharmacia) as described by Valenta et al.<sup>15</sup> For semiquantitative estimation of specific IgE against total timothy grass pollen proteins and purified recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, and rPhl p 5) an ELISA was used. Amounts of allergens and serum dilutions were used so that antigens were in excess of IgE and IgG antibodies to allow the determination of the relative percentage of grass pollen-specific antibodies directed against the recombinant allergens. The optimal amounts of allergens and serum dilutions were determined in pilot experiments.

ELISA plates were coated with 50 µg/ml total timothy grass pollen proteins or purified, recombinant allergens (2 µg/ml). The plates were washed two times with phosphate-buffered saline (PBS), 0.05% Tween-20, blocked for 2.5 hours with PBS, 1% BSA, 0.05% Tween-20 at room temperature and incubated overnight at 4° C with sera from patients with grass pollen allergy. Patients' sera were diluted 1:20 in PBS, 0.5% BSA, 0.05% Tween-20 for the detection of specific IgE and 1:100 for detection of IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub>. The plates were washed five times with PBS, 0.05% Tween-20, and bound immunoglobulins were detected with monoclonal mouse anti-human Ig antibodies (PharMingen, San Diego, Calif.), diluted 1:1000 in PBS, 0.5% BSA, 0.05% Tween-20 overnight at 4° C. The plates were washed five times with PBS, 0.05% Tween-20 and

incubated for 30 minutes at 37° C and 30 minutes at 4° C with a horseradish-peroxidase-coupled sheep anti-mouse antiserum (Amersham, Buckinghamshire, U.K.), diluted 1:2000 in PBS, 0.5% BSA, 0.05% Tween-20. Plates were again washed five times with PBS, 0.05% Tween-20, and ABTS (60 mmol/L citric acid, 77 mmol/L Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1.7 mmol/L ABTS [Sigma, St. Louis, Mo.], 3 mmol/L H<sub>2</sub>O<sub>2</sub>) was added. Plates were incubated in the dark for 30 minutes at room temperature, and the color reaction was stopped by addition of 100 µl/well 0.32% NaF. Extinctions (optical density 450 to 405 nm) were determined with an ELISA reader (Dynatech, Denkendorf, Germany).

#### **In vitro histamine release assays with purified recombinant allergens**

Heparinized blood samples were obtained from patients with grass pollen allergy after informed consent had been given. Granulocytes were prepared by dextran sedimentation and washed and incubated with various doses of recombinant allergens, anti-human IgE, or buffer as previously described.<sup>16</sup> Liberated histamine was measured in the cell-free supernatants by radioimmunoassay (Immunotech, Marseille, France).

### **RESULTS**

#### **Expression in *E. coli* and purification of recombinant timothy grass pollen allergens**

In a previous study recombinant timothy grass pollen allergens were expressed as β-galactosidase fusion proteins.<sup>9</sup> In an attempt to obtain recombinant nonfusion allergens, which are similar to the natural allergens, polymerase chain reaction primers were synthesized according to the Phl p 1, Phl p 2, and Phl p 5 cDNA sequences to allow the amplification of cDNAs without the hydrophobic leader sequences that are absent in the mature proteins. The cDNAs coding for the mature allergens were amplified from the allergen cDNAs and subcloned into plasmid pET. Recombinant nonfusion proteins were expressed in *E. coli* BL21 (DE3) on induction with IPTG and purified. Fig. 1 shows a Coomassie blue-stained protein gel containing total timothy grass pollen extract in lane P and purified recombinant timothy grass pollen allergens (lanes I, II, and V). Milligram amounts of more than 90% pure and water-soluble recombinant Phl p 1, Phl p 2, and Phl p 5 were obtained for subsequent immunologic testing.

#### **IgE-binding capacity of recombinant timothy grass pollen allergens**

Purified recombinant timothy grass pollen allergens were tested for their antibody-binding capacity in denaturing and nondenaturing assays. Nitrocellulose-blotted natural timothy grass pollen



FIG. 1. Coomassie brilliant blue-stained polyacrylamide gel. Lane M represents a molecular weight marker; lane P shows a protein extract from timothy grass pollen; lane I, purified rPhl p 1; lane II, purified rPhl p 2; and lane V, purified rPhl p 5.

allergens and purified recombinant timothy grass pollen allergens were compared for IgE binding by using sera from 13 patients with grass pollen allergy. The sera were selected to represent the typical IgE-binding pattern to timothy grass pollen allergens from 100 individuals with grass pollen allergy as determined by case history, RAST, and skin prick testing.<sup>7,9</sup> With the exception of patient 6 (Fig. 2), all patients showed IgE reactivity with nitrocellulose-blotted natural timothy grass pollen allergens. IgE binding was observed in the 30 kD range with group I and group V allergens and at around 10 kD with group II allergens. Two patients (nos. 2 and 4) showed weak reactivity to group IV allergens at 54 kD. All patients, including patient 6 who had no detectable IgE against natural timothy grass pollen allergens, displayed IgE reactivity to recombinant Phl p 1. Twelve of 13 patients reacted with recombinant Phl p 5, and 10 patients had IgE against recombinant Phl p 2. Recombinant timothy grass pollen allergens thus allowed differentiation among IgE reactivity to group I, group II, and group V allergens, which cannot be distinguished when nitrocellulose-blotted natural grass pollen extracts are used.

The ELISA results (Fig. 3) showed that in most of the patients high levels of timothy-specific IgE were directed against Phl p 5 and Phl p 1. Phl p 2 bound rather low levels of specific IgE. The reactivity in the different IgG subclasses with a given allergen was not always associated with its IgE-binding capacity (Fig. 3).

#### Recombinant timothy grass pollen allergens induce specific and dose-dependent histamine release

To demonstrate specific histamine release capacity of recombinant timothy grass pollen aller-

gens, basophils from patients with grass pollen allergy were incubated with recombinant Phl p 1, Phl p 2, or Phl p 5. Figs. 4 and 5 show that purified recombinant timothy grass pollen allergens induced specific and dose-dependent histamine release in patients with grass pollen allergy. To ensure that specific IgE antibodies were present in the patients' sera at the time when the histamine release assays were done, supernatants obtained during the enrichment of basophils were probed for IgE reactivity with natural and recombinant timothy grass pollen allergens (Figs. 2 and 3). Comparing the IgE reactivity of the patients displayed in the immunoblots in Fig. 2 with the histamine release capacity of recombinant allergens, we found that all patients with IgE antibodies specific for the recombinant allergen tested also had positive histamine release results. The optimal dose of recombinant timothy grass pollen allergens for inducing maximal histamine release was usually approximately 1  $\mu$ g/ml. As in previous studies performed with recombinant fusion allergens and natural allergens, histamine release was observed at doses as low as 10 ng/ml allergen.<sup>6</sup> The recombinant allergens were also tested with basophils from patients without specific IgE antibodies and were found to induce no histamine release up to concentrations of 10  $\mu$ g/ml recombinant allergen (data not shown). Negative controls performed without addition of recombinant allergens or recombinant allergens against which patients were not sensitized did not lead to histamine release, whereas by using a monoclonal anti-human IgE antibody (positive control), histamine release could be induced in all patients tested as previously described.<sup>16</sup>

#### DISCUSSION

A number of cDNAs coding for plant, mite, animal, and fungal allergens have been characterized.<sup>17,18</sup> Many of these cDNAs were obtained by IgE immunoscreening of expression cDNA libraries,<sup>18</sup> and it was thus possible to test the bacterially expressed allergens for their IgE-binding capacity with sera from allergic patients. In previous studies the molecular cloning and expression of cDNAs coding for three major timothy grass pollen allergens (Phl p 1, Phl p 2, and Phl p 5) has been reported.<sup>4-6</sup> With  $\beta$ -galactosidase-fused recombinant timothy grass pollen allergens it was shown that a panel of only three to four recombinant timothy grass pollen allergens can be sufficient to diagnose grass pollen allergy.<sup>7</sup> In another study it has been demonstrated that a combination of these

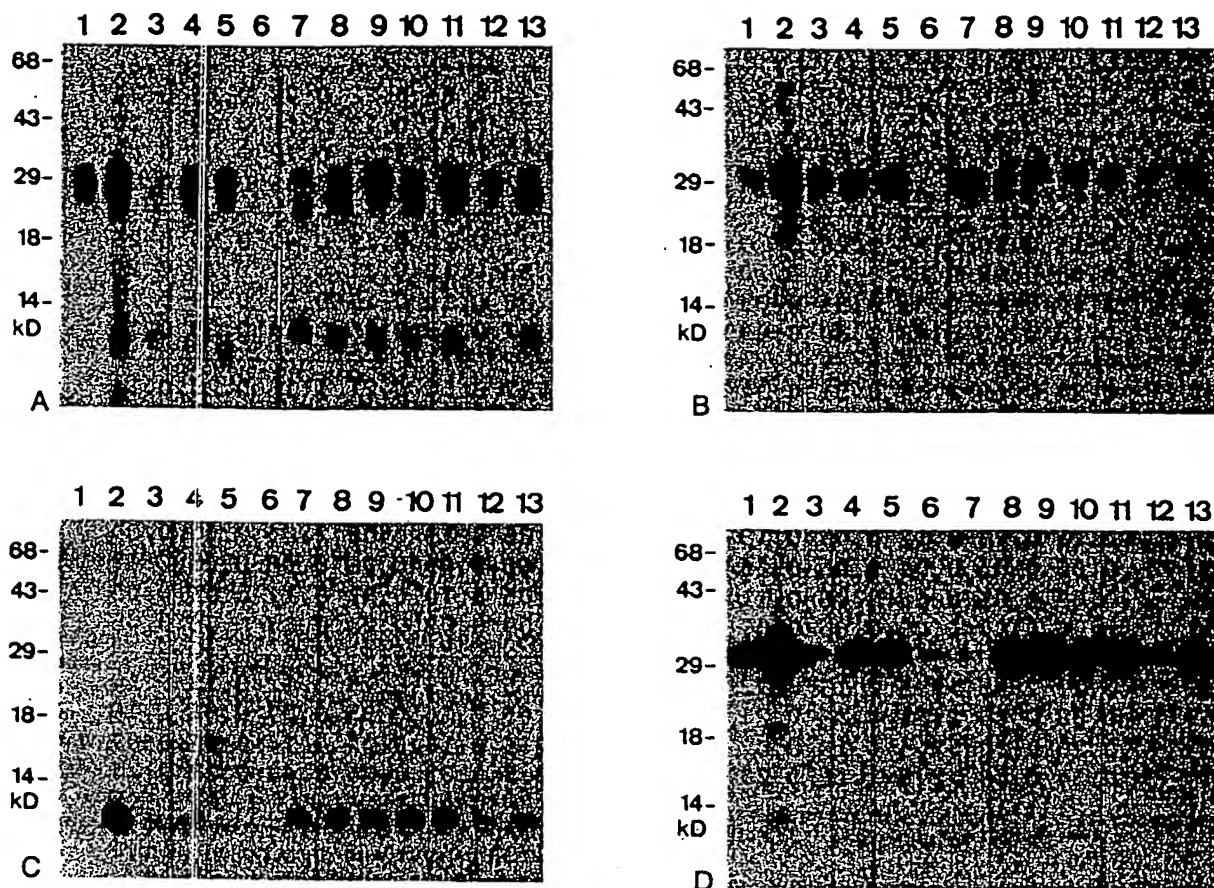


FIG. 2. IgE immunoblots with total timothy grass pollen proteins (A), purified rPhl p 1 (B), purified rPhl p 2 (C), and purified rPhl p 5 (D). Thirteen patients with timothy grass pollen allergy (lanes 1 to 13) were tested for IgE binding to nitrocellulose-blotted natural timothy grass pollen proteins (A) and purified recombinant timothy grass pollen allergens (B, C, and D).

three recombinant timothy grass pollen allergens bound a high percentage of grass pollen-specific IgE.<sup>9</sup> Although  $\beta$ -galactosidase-fused timothy grass pollen allergens showed similar IgE-binding capacity compared with natural timothy grass pollen allergens, the expression of large amounts of rPhl p 1, rPhl p 2, and rPhl p 5 without leader peptides and fused protein was attempted. In this study the expression of functional rPhl p 1, rPhl p 2, and rPhl p 5 is reported. The recombinant allergens lack their hydrophobic leader peptides and other additionally fused polypeptides. In contrast to the natural allergens, an additional methionine is present at the N-terminus, and no carbohydrates or hydroxyprolines are present in the recombinant proteins because of the bacterial expression system used. As already demonstrated for the  $\beta$ -galactosidase-fused allergens, IgE binding to the native and denatured allergens was shown. The use of rPhl p 1, rPhl p 2, and rPhl p 5 allowed a precise determination of the

patients' sensitization patterns. A major advantage of recombinant allergens is the possibility to discriminate patients' IgE reactivity to allergens of a similar molecular weight, such as Phl p 1 or Phl p 5. Patient 6, who showed no detectable IgE reactivity to nitrocellulose-blotted natural grass pollen extract, was found to react with recombinant Phl p 1. Although considerable amounts of natural grass pollen extracts had been used to coat the ELISA plates, a combination of the three recombinant timothy grass pollen allergens yielded, for the most part, a higher extinction than the natural extract. Recombinant allergens therefore offer the advantage of measuring allergen-specific antibody levels, and it appears that carbohydrate moieties or amino acid modifications, such as hydroxyprolines, which frequently occur in natural grass pollen allergens, are not of relevance for patients' IgE binding.

In addition to the antibody-binding capacity, the biologic activity of rPhl p 1, rPhl p 2, and rPhl p 5

#	1	2	3	4	5	6	7	8	9	10	11	12	13	
E	0.16	2.26	0.18	0.40	0.44	0.05	0.36	0.34	0.88	0.22	0.78	0.16	0.24	IgE
I	0.10	0.52	0.11	0.10	0.29	0.05	0.29	0.13	0.47	0.13	0.16	0.09	0.10	
II	0.08	0.19	0.09	0.05	0.05	0.04	0.12	0.10	0.13	0.06	0.12	0.05	0.07	
V	0.17	1.54	0.14	0.43	0.34	0.05	0.06	0.32	1.33	0.18	0.99	0.16	0.24	
E	0.15	0.60	0.50	0.28	0.20	0.09	0.16	0.73	0.20	0.13	0.47	0.26	0.25	IgG1
I	0.08	0.28	0.27	0.11	0.10	0.10	0.12	0.58	0.18	0.11	0.10	0.16	0.20	
II	1.09	0.25	0.31	0.09	0.07	0.12	0.38	0.38	0.10	0.15	0.08	0.19	0.13	
V	0.10	0.45	0.48	0.30	0.09	0.10	0.10	0.81	0.65	0.16	0.14	0.25	0.19	
E	0.23	0.63	1.19	0.51	0.34	0.14	0.20	1.77	0.50	0.21	1.18	0.28	0.14	IgG2
I	0.23	0.50	0.64	1.13	0.61	0.94	0.64	0.55	1.04	0.24	1.32	1.16	0.36	
II	1.01	0.19	0.48	0.47	0.29	0.34	0.24	0.58	0.71	0.44	0.54	0.54	0.46	
V	0.14	0.20	0.66	0.25	0.21	0.12	0.14	1.25	0.83	0.18	0.37	0.47	0.13	
E	0.11	0.28	0.12	0.08	0.13	0.15	0.12	0.35	0.13	0.08	2.04	0.07	0.09	IgG3
I	0.06	0.05	0.05	0.05	0.06	0.13	0.09	0.08	0.09	0.05	0.08	0.05	0.07	
II	0.09	0.05	0.06	0.06	0.11	0.06	0.04	0.05	0.70	0.37	0.11	0.05	0.05	
V	0.26	0.07	0.05	0.05	0.06	0.11	0.05	0.09	0.08	0.28	0.07	0.58	0.05	
E	0.27	1.95	>2.5	2.15	0.22	0.06	0.28	>2.5	0.29	0.75	0.42	0.13	0.14	IgG4
I	0.08	0.40	2.31	0.36	0.21	0.06	0.18	2.05	0.16	0.35	0.27	0.08	0.10	
II	0.20	0.50	>2.5	0.19	0.07	0.07	0.12	>2.5	0.17	0.27	0.14	0.06	0.07	
V	0.14	0.58	>2.5	1.10	0.20	0.05	0.06	>2.5	0.33	0.54	0.48	0.10	0.15	

FIG. 3. IgE, IgG<sub>1</sub>-IgG<sub>4</sub> reactivity to natural (E) and recombinant timothy grass pollen allergens (I, Phl p 1; II, Phl p 2; V, Phl p 5) estimated by ELISA in 13 patients with grass pollen allergy. Extinctions over baselines determined with a group of nonallergic individuals are displayed.

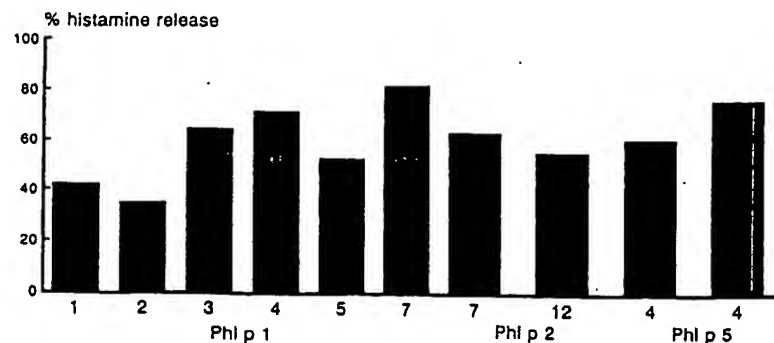


FIG. 4. Induction of histamine release from basophils of patients with grass pollen allergy with purified recombinant Phl p 1, Phl p 2, and Phl p 5. Basophils from six patients allergic to Phl p 1 were incubated with recombinant Phl p 1; basophils from two donors were incubated with recombinant Phl p 2 and basophils from one patient allergic to Phl p 5 (two experiments with the same patient). The percentage of histamine release at the optimal allergen concentration (1  $\mu$ g/ml) is displayed. Patient numbers are the same as in Fig. 2 to allow a comparison of histamine-releasing capacity and IgE-binding capacity of recombinant timothy grass pollen allergens.

was demonstrated in basophil degranulation assays. The basophil degranulation assay<sup>19</sup> has been shown to closely reflect the cascade of the allergic effector reaction, and in the case of recombinant birch pollen allergens, has already served as a valuable tool for testing recombinant allergens in a

close to in vivo system.<sup>15, 19</sup> In this study purified recombinant timothy grass pollen allergens induced dose-dependent and specific histamine release from basophils of sensitized patients. Even at concentrations up to 10  $\mu$ g/ml, no cell toxic effects were observed.



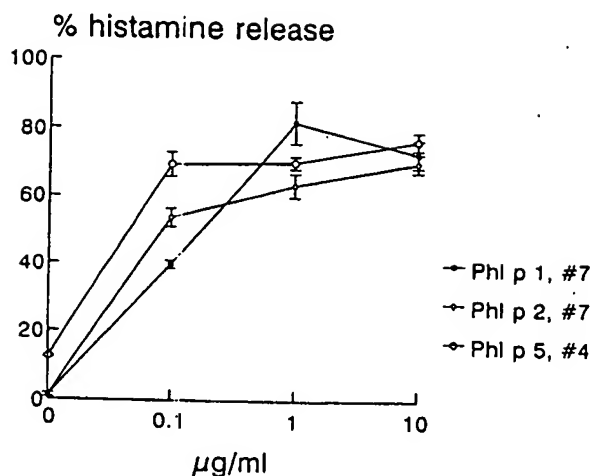


FIG. 5. Dose dependency of histamine release induced with basophils from representative patients allergic to grass pollen by use of recombinant Phl p 1, Phl p 2, and Phl p 5. Basophils from three representative patients with grass pollen allergy were incubated with different concentrations of recombinant timothy grass pollen allergens, and histamine release was determined in the cell-free supernatants by radioimmunoassay. Histamine release induced from basophils of patient 7 with rPhl p 1 and rPhl p 2 and the dose dependency of a histamine release obtained with recombinant Phl p 5 in patient 4 are shown. Patient numbers are the same as in Fig. 2.

In conclusion we believe that the expression of large amounts of recombinant timothy grass pollen allergens will substantially add to current forms of allergy diagnosis by allowing determination of the patients' sensitization patterns (allergogram). The recombinant nonfusion allergens can be produced in gram amounts with consistent batch-to-batch quality and can be used for current in vitro allergy tests, as well as for skin prick testing. On the basis of the determination of the patients' individual sensitization patterns and specific antibody levels, immunotherapy with selected recombinant allergens is a realistic prospect for the near future.<sup>20</sup>

## REFERENCES

- Freidhoff LR, Ehrlich-Kautzky E, Grant JH, Meyers DA, Marsh DG. A study of the human immune response to *Lolium perenne* (rye) pollen and its components, *Lol p I* and *Lol p II* (rye I and rye II). *J ALLERGY CLIN IMMUNOL* 1986;78:1190-201.
- Ansari AA, Shenbagamurthi P, Marsh DG. Complete primary structure of a *Lolium perenne* (perennial rye grass) pollen allergen, *Lol p III*: comparison with known *Lol p I* and *II* sequences. *Biochemistry* 1989;28:8665-70.
- Matthiesen F, Loewenstein H. Group V allergens in grass pollens. II. Investigation of group V allergens in pollens from 10 grasses. *Clin Exp Allergy* 1991;21:309-20.
- Laffer S, Valenta R, Reimitzer I, et al. Complementary DNA cloning of the major allergen *Phl p I* from timothy grass (*Phleum pratense*); recombinant *Phl p I* inhibits IgE binding to group I isoallergens from eight different grass species. *J ALLERGY CLIN IMMUNOL* 1994;94:689-98.
- Dolecek C, Vrtala S, Laffer S, et al. Molecular characterization of *Phl p II*, a major timothy grass (*Phleum pratense*) pollen allergen. *FEBS Lett* 1993;335:299-304.
- Vrtala S, Sperr WR, Reimitzer I, et al. cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen: characterization of the recombinant *Phl p V* allergen. *J Immunol* 1993;151:4773-81.
- Valenta R, Vrtala S, Ebner C, Kraft D, Scheiner O. Diagnosis of grass pollen allergy with recombinant timothy grass (*Phleum pratense*) pollen allergens. *Int Arch Allergy Immunol* 1992;97:287-94.
- Valenta R, Ball T, Vrtala S, Duchêne M, Kraft D, Scheiner O. cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. *Biochem Biophys Res Comm* 1994;199:106-18.
- Laffer S, Vrtala S, Duchêne M, et al. IgE-binding capacity of recombinant timothy grass (*Phleum pratense*) pollen allergens. *J ALLERGY CLIN IMMUNOL* 1994;94:88-94.
- Susani M, Jertschin P, Dolecek C, et al. High level expression of birch pollen profilin (*Bet v II*) in *Escherichia coli*: purification of the recombinant allergen and evaluation of the IgE-binding capacity (Submitted for publication).
- Way M, Pope B, Gooch J, Hawkins M, Weeds AG. Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J* 1990;9:4103-9.
- McLeod M, Stein M, Beach D. The product of the mei<sup>+</sup> gene, expressed under the control of the mating-type locus, induces meiosis and sporulation in fission yeast. *EMBO J* 1987;6:729-36.
- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989.
- Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 1990;185:60-89.
- Valenta R, Duchêne M, Ebner C, et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992;175:377-85.
- Valent P, Besemer J, Muhm M, Majdic O, Lechner K, Bettelheim P. Interleukin 3 activates human blood basophils via high-affinity binding sites. *Proc Natl Acad Sci USA* 1989;86:5542-6.
- Scheiner O, Bohle B, Breitenbach M, et al. In: Godard P, Bousquet J, Michel FB, eds. Recombinant allergens: production and possible clinical implications. Advances in allergology and clinical immunology. Carnforth, England: The Parthenon Publishing Group, 1992:115-27.
- Bousquet J, Valenta R. *In vivo* and *in vitro* use of recombinant allergens. *ACI News* 1994;6/2:54-9.
- Valenta R, Sperr WR, Ferreira F, et al. Induction of specific histamine release from basophils with purified natural and recombinant birch pollen allergens. *J ALLERGY CLIN IMMUNOL* 1993;91:88-97.
- Valenta R, Duchêne M, Vrtala S, et al. Recombinant allergens as candidates for immunotherapy of type I allergic diseases. In: Chanock RM, Ginsberg HS, Brown F, Lerner R, eds. Vaccines 93. New York: Cold Spring Harbor Laboratory Press, 1993:37-42.

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Linhart, *et al.*

Appl. No. 10/026,914

Filed: December 27, 2001

For: Allergy Vaccines Containing Hybrid Polypeptides

Art Unit: 1645

Examiner: Jana A. Hines

Any. Docket: 966927.00006 (0273-0006)

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

1. Professor Rudolf Valenta, am an associate professor at the Division of Immunopathology (Department of Pathophysiology, University of Vienna, Austria), and I do hereby declare and state that:
  1. I studied medicine at the University of Vienna and graduated with an MD degree in 1987.
  2. Since 1988 I have been working at the Department of Pathophysiology (formerly: Department of General and Experimental Pathology) at the University of Vienna, Austria.
  3. I extended my scientific experience during trainings in molecular biological techniques in the labs of Markus Susani (Institute of Molecular Biology, Salzburg, Austria) and Michael Brettenbach (Department of Genetics and Microbiology, University of Vienna) in 1988.
  4. In 1992, I was awarded the qualification of a University lecturer for general and experimental pathology and became Head of the Molecular Immunopathology group at the Division of Immunopathology.
  5. After a training in cellular mouse immunology in the lab of Alec Sehon (Department of Immunology, University of Manitoba, Winnipeg, Canada) and a one year's specialist training in Internal medicine and design of clinical studies at the Department of Hematology and the Department of Clinical Pharmacology of the University of Vienna in the years 1994 and 1995, I was granted a specialist degree for Pathophysiology in 1996.
  6. In 1997, I completed my specialist training in immunology and became associate professor for Pathophysiology.



7. I have been Head of the Division of Immunopathology since December 2001 and have since headed the special research program: Molecular and immunological strategies for prevention, diagnosis and treatment of Type I allergies.

8. I have contributed to more than 200 peer-reviewed scientific papers, reviews, and book Chapters.

9. My work on the characterization of allergens and their use for new concepts of allergy treatment was granted numerous scientific awards, among others: the Sandoz Austria Award for Biology in 1994; the International Award of the Pharmacia Allergy Research Foundation in 1996; the START Award of the Austrian Science Fund in 1998; and the Sarstedt Science Award in 2000.

10. I have read and understood the Examiner's basis for rejection of the claims of the above-captioned Application, in the June 15, 2006, Office Actions particularly the 35 U.S.C. § 103(a) rejection over Ball et al., (WO 95/34578) in view of Vrtala et al., (1996: J. Allerg Clin. Immun. Vol 97(3): 781-787).

11. I have read and understood the disclosure and teachings of the Ball et al. reference (Same as US 6,008,340) particularly in reference to fusion polypeptides of Phl p1 epitopes and one other additional polypeptide.

12. Essentially Ball et al. teaches the use of "fusion polypeptides of Phl p1 epitopes and one other polypeptide," wherein that other polypeptide is used to drive the expression process and purification process. (See US 6,008,340 Col. 3, lines 1-6; Col. 5, lines 59-62; and Col. 8, lines 15-31).

13. Ball et al. failed to teach or suggest that the fusion polypeptide of Phl p1 epitopes and another polypeptide that can be expressed as a fusion protein in prokaryotic or eukaryotic cells can be used as an immunotherapeutic agent.

14. In fact, Ball et al. failed to teach or suggest the fusion polypeptide of Phl p1 epitopes with a second polypeptide, which is itself an allergen.

15. In fact, Ball et al. failed to teach or suggest the fusion polypeptide of Phl p1 epitopes to a second, third, fourth etc polypeptide, all of which are allergens.

16. In contrast, the present invention teaches that one or more recombinant produced timothy grass pollen allergens can be fused and said fusion protein can be used as immunotherapeutic agent and moreover that the immunogenicity of each of the components of the fusion protein are increased through the fusion described in the present invention.

17. In fact, the inventors are surprised that fusion proteins of naturally occurring allergens can be used as immunotherapeutic agents and exhibit increased immunogenicity.

18. This surprising result is the subject of much speculation as to whether the fusion of allergens in some way destroys certain epitopes in the fusion allergen compared to the native unfused allergens.

19. To validate this surprising discovery, we tested whether immunization with the fusion allergens induces IgG antibodies (IgE-blocking antibodies) that recognize the individual allergen components.

20. As demonstrated in Figure 5 of the instant specification, the average IgG1 responses induced by the hybrid molecules to each of the individual allergens (rPhl p1, rPhl p2, rPhl p5, rPhl p6) were higher than those obtained by immunization with the single allergen components.

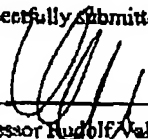
21. To further validate this surprising discovery, we tested whether mouse antibodies induced with the hybrid molecules can block the binding of grass pollen allergic patient's IgE antibodies to purified grass pollen allergens.

22. As shown in Tables 3A and 3B of the instant specification, IgG antibodies induced with the rP2-P6 and the rP6-P2 fusion proteins caused a 48%-34% inhibition of IgE binding to Phl p2 and a 54% to 67% inhibition of IgE binding to Phl p6 (Table 3A). By contrast, the inhibition of IgE reactivity yielded by preincubation with antibodies induced with rPhl p2 and rPhl p6 alone was very low (0-15%). (Table 3A).

All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents ensuing thereon.

Date: 22. June, 2006

Respectfully submitted,

  
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